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VITAMINS AND HORMONES

VOLUME VIII

VITAMINS AND HORMONES

ADVANCES IN RESEARCH AND APPLICATIONS

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VOLUME VIII

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EDITORS' PREFACE

By this time *Vitamins and Hormones* needs no special introduction to the scientific public. The success of seven previous volumes can only mean that the series serves a useful purpose in surveying, reporting and assessing the progress of the rather broad field which it covers.

The present volume continues the general trend already set. The field shows no signs of contracting, and indeed the number of workers in it appears to be increasing steadily. Recent developments in medicine will probably lead to still further increases.

The critical and thorough nature of the reviews in the present volume testifies to the very large investment of time and trouble on the part of the authors, as well as to their familiarity with an extensive and scattered literature. The Editors have been fortunate in having such distinguished contributors, and take this opportunity of formally expressing their thanks.

KENNETH V. THIMANN
ROBERT S. HARRIS

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"Animal Protein Factor" and Vitamin B_{12} in the Nutrition of Animals

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I. BACKGROUND AND OUTLINE OF RECENT PROGRESS

It is difficult to say when or where the thought first arose that there may be a nutritional quality associated with animal protein which is not shared by vegetable food sources and is not dependent on amino acid make up. The idea was put into words by Mapson (1932, 1933) and by Byerly *et al.* (1933). The work of the former dealt with rats fed diets containing special purified caseins; it was almost overlooked at the time and even later did not receive the attention it deserved because of certain difficulties of interpretation. The more recent studies of Cary and Hartman re-establish Mapson as a pioneer in this field. Unfortunately Mapson did not continue these studies. Byerly's group (Office of Poultry Investigations, Beltsville) carried on and laid the groundwork both for more rational studies with vegetable proteins and for highly significant advances towards the isolation of the unknown factor.

As a period of scarcity of animal protein sources came on, with general wartime shortages, soybean meal was introduced into poultry feeding on a much larger scale. The unavailability of animal protein supplements made necessary an intensified study of both the slight amino acid deficiencies of soybeans (principally methionine) and the postulated unknown factor. From 1946 on, the poultry literature bristled with papers on various aspects of this problem. These will not be cited here but most of them will be referred to later on. A perusal of the table of contents of *Poultry Science* for 1946 and 1947 will give the reader a picture of both the intensity of effort and the types of problems which had to be solved. For scientific purposes it was necessary step by step to differentiate the effects of various vitamin and amino acid deficiencies and for practical purposes to find suitable sources of the unknown factor. The main achievements will be summarized here. It was firmly established that an unknown factor not present in any vegetable feeds was a determinant in the quality of various animal protein supplements. Meat scrap was rated lower and fish meal higher than heretofore and a new product "fish solubles"[1] was introduced and shown to be very potent and apparently more dependable than many commercially available animal by-products in use. Liver and liver extracts were proved to be high potency sources for experimental work. The factor in question came to be known as "animal protein factor" and later the term "animal protein factor complex" reflected the conviction that more than one substance was involved.

The results of Bird and associates at Beltsville should be specifically mentioned here since they gave a new turn to the investigation. Hammond (1944) had shown earlier that cow manure contained very appreciable amounts of B factors and could find application as a substitute for alfalfa. Bird and coworkers (Rubin and Bird, 1946a, b) demonstrated that cow manure also carried the factor to which fish meal owed its distinctive value for hatchability and growth of chicks and gave methods for concentrating the factor a thousand fold (Bird, 1947b). Bird's additional finding that the factor occurred in hen droppings (Rubin *et al.*, 1946) was further developed by McGinnis *et al.* (1947a) who showed that the fresh droppings were practically inactive but on incubation high potency developed. These findings gave assurance that the factor was not necessarily dependent on feeding animal tissues but had an independent microbiological origin.

In the meantime, Cary and Hartman, without reference to the poultry investigations, picked up again the thread of Mapson's work. In a series of preliminary communications and short government reports (Bureau of Dairy Industry, U.S. Dept. Agriculture) from 1941 on, they showed that by extraction with hot alcohol a factor that is a nutritional essential for rats can be removed from crude casein. Liver and other material including dairy products were successful supplements.

Zucker and Zucker worked with rats on an all-plant diet progressively supplemented with more and more known factors. The resulting deficiency was relieved by liver or crude casein and not by yeast, a distribution of activity which caused them, in 1946, to suggest identity of their factor with Cary's. Activity was then established for the two recently announced successful poultry supplements—Bird's cow manure concentrate and fish solubles—leading to the suggestion in 1947 that a similar deficiency could occur in both birds and mammals induced either by the removal of a factor from the diet materials, as in Cary's work, or by feeding a naturally deficient diet. Active supplements were the same for rats and chicks.

---

[1] Description of, and analytical data on, this product are given by Lassen and Bacon (1946) and by Mishler *et al.* (1948).

The isolation of a nutritional factor in pure form is always a significant turning point and opens new paths. This is what happened when from the laboratories of Merck and Co. in April, 1948 (Rickes *et al.*, 1948a) the announcement came that a red crystalline product had been isolated from liver which, in clinical tests carried out by West (1948), proved to be the antipernicious anemia substance. Almost simultaneously the laboratories of Glaxo House in England announced a red cobalt-containing product from liver with antipernicious anemia activity in microgram quantities (Smith, 1948a, b). Closely upon this followed communications from the Merck Therapeutic Institute showing that the new compound called B_{12} had both in rats and in chicks the properties of an "animal protein factor" (Emerson, 1948; Ott *et al.*, 1948). A microbiological origin for B_{12} was demonstrated when the Merck group announced the large scale preparation of the identical material from cultures of *Streptomyces griseus* (Rickes *et al.*, 1948c). This was somewhat preceded by production of clinically active concentrates from bacterial cultures from hen feces at the Lederle Laboratories (Stokstad *et al.*, 1948). About this same time a project was undertaken at the Western Regional Research Laboratories in collaboration with McGinnis (Stephenson *et al.*, 1948; McGinnis *et al.*, 1949) involving the microbiological production of active material from a number of organisms including as common a one as *Bacillus subtilis.*

The isolation work of the Merck group was made possible by a microbiological technique devised by Shorb (1947, 1948) using *Lactobacillus lactis* Dorner as test organism. Later *L. leichmannii* was also used for assay purposes (Skeggs *et al.*, 1948; Hoffman *et al.*, 1948).

Before we proceed to a detailed consideration it should be realized that progress was not as simple as it may seem from an outline given after the event. The microbiological assays rendered service efficient enough in isolation work but have otherwise presented knotty problems. Standardization of the material for pharmaceutical purposes is still uncertain. In the poultry field, which was really the proving ground for the nutritional phase, it was well known that poultry has a high and exigent requirement for amino acids and much attention had to be given to this phase of the protein problem. During practically the whole of the time of development of "animal protein factor" work, the literature abounded also in statements such as "animal protein supplements are not essential in chick rations for rapid growth." How rapidly a complete change in attitude set in is illustrated by the fact that the man whose chicks in 1948 furnished the first proof that crystalline B_{12} has "animal protein factor" activity published in 1944 under the title: "Satisfactory early chick growth on a diet containing no animal protein."

The answer to the riddle of why chicks do and do not need B_{12} lies largely in two circumstances characteristic of B_{12}: an unusually effective storage in the maternal organism for transmissal to the offspring and the considerable production of B_{12} activity in the feces, making it available to the young unless access is prevented.

II. CHEMISTRY OF VITAMIN B_{12}

1. The Crystalline Substance

Crystalline material has been obtained by investigators at Merck, Glaxo Laboratories, British Drug Houses, the Lederle Laboratories, and E. R. Squibb and Sons. Both British groups have had preparations of a substance believed to be identical with the Merck B_{12} (Brink *et al.*, 1949a; Smith, 1949). Accepting this as so, data will be reported from all three groups on the properties of B_{12}.

The red crystals, said to be hygroscopic (Merck and Co., 1949) darken at 190–215°, and do not melt below 300°C. (Rickes *et al.*, 1948c; Brink *et al.*, 1949a). Refractive indices of the crystals are (α) 1.616; (β) 1.652, (γ) 1.664 (Rickes *et al.*, 1948c). Data for solubility are fragmentary; it is soluble in H_2O at least to the extent of 26.3 mg. in 15 ml. (Kaczka *et al.*, 1949). It is crystallized by the addition of four or more parts of acetone to an aqueous solution. The absorption spectrum in water is characterized by maxima at 2780 A.($E^{1\%}_{1\text{cm.}} = 115$); 3610 A.(204); 5500 A.(63) and is not much affected by change in pH (Brink *et al.*, 1949a). An absorption curve has been published by Ellis *et al.* (1949a), and is said to differ only in fine detail from that of the Merck group (Brink *et al.*, 1949a). This spectrum is stated to be incompatible with the presence of any pterin group in the molecule. B_{12} is optically active, with $[\alpha]^{23°}_{6563\text{A.}} = -59 \pm 9°$, and is a polyacid base, whose basic groups are too weak to show up in potentiometric titration in aqueous solution, but can be demonstrated in glacial acetic acid (*ibid.*).

The empirical formula is given as $C_{61-64}H_{86-92}N_{14}O_{13}PCo$ (*ibid.*). The presence of Co and P was first reported by Smith (1948b). The minimum molecular weight based on a molecule containing 1 atom of Co (Co concentration being 4.5%) is 1300 (Brink *et al.*, 1949a). A rough ebullioscopic determination in methanol indicates a molecular weight of 1490 ± 150 but the value based on Co content is preferred (Brink *et al.*, 1949a; Wolf *et al.*, 1949). X-ray studies indicate an approximate molecular weight of 1550–1750 (Smith, 1948b). The compound is characterized as a cobalt coordination complex with six groups around the cobalt, and the red color is attributed to this structural feature (Rickes *et al.*, 1948b). No statement has appeared as to whether the Co complex in B_{12} carries a net charge; i.e., whether the groups attached to the Co carry enough negative charges to neutralize the charge on the Co. The stated molecular weight from the boiling point elevation is apparently based on the assumption that B_{12} does not dissociate in solution. The state of oxidation of the Co has not been reported. B_{12} is subject to reduction by hydrogen in the presence of a catalyst, with accompanying change in color from red to brown, and the reduced product spontaneously oxidizes in air (Kaczka *et al.*, 1949). These facts would fit a tervalent Co in B_{12}, although other interpretations might be made since it is not demonstrated that the Co was specifically involved in the reduction.

Michaelis (1948) has recently considered the properties of Co complexes which show reversible oxygenation, i.e., the addition of an O_2 molecule. The Co is in the divalent state and the oxygenation results in a binuclear cobaltous complex, with O_2 as a bridge between two cobalts. If B_{12} has, as stated, only 1 Co per molecule and if this should also be true for all pertinent derivatives, then of course these considerations do not apply. The redox properties of B_{12} and derivatives are of particular interest in view of the relation between degree of oxygenation of the medium and the requirement for B_{12} of the lactobacilli (see Section V, 1).

B_{12} is stable in neutral aqueous solution at room temperature for over a year (Merck and Co., 1949) and a solution of 74 μg./.5 ml. withstands autoclaving for 15 minutes at 121°C. (Rickes *et al.*, 1948b). In .01 *N* HCl at room temperature a solution of 10 μg./ml. has lost 18% of its potency after 3 hours, 75% after 23 hours (*ibid.*). In .015 *N* NaOH a solution of 0.2 μg/ml. deteriorates somewhat more rapidly, losing 20% activity after ⅔ hour, 45% after 6 hours, 90% after 23 hours (*ibid.*). According to Smith (1948a), boiling in alkaline solution discharges the red color. Boiling with normal HCl for 1 hour does not apparently alter the color, but leads to the formation of two pigments, both insoluble in ether as is B_{12}, but one extractable with $CHCl_3$ the other with *n*-butanol (*ibid.*). The P can be hydrolyzed off in 20% HCl at 100° after about 6 hours, although no liberation of phosphate is detected at room temperature after 17 days (Ellis *et al.*, 1949b). A product of this reaction is a black Co complex with a visible spectrum (in dioxane) similar to that of B_{12}. It is insoluble in water but soluble in dilute alkali (Ellis *et al.*, 1949a).

Pyrolysis and alkaline fusion yield some evidence for the presence of pyrroles or other N-containing 5-membered rings (Brink *et al.*, 1949a). "Degradation of vitamin B_{12} by acid hydrolysis" yields a product identified as 5,6-dimethylbenzimidazole, which is thought to be bound to the rest of the molecule according to Fig. 1 (Brink *et al.*, 1949b).

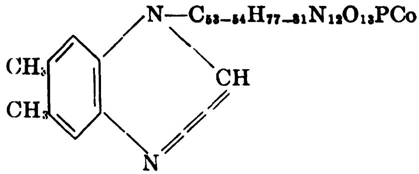

FIG. 1.

While crystalline preparations have yielded evidence of small amounts of alpha amino acids liberated by acid hydrolysis, in very well purified samples no amino acids can be detected by paper chromatography, and are presumably present to less than 0.2% (Brink *et al.*, 1949a).

Two other biologically active compounds besides B_{12} have been characterized—B_{12a} and B_{12b}. B_{12a} (Kaczka *et al.*, 1949) is formed on exposure to air of a solution of B_{12} that has been catalytically reduced. It is a red crystalline compound differing only slightly in physical properties from B_{12}, but with only $\frac{1}{6}$ to $\frac{1}{2}$ of the biological activity (*ibid.;* Wolf *et al.*, 1949; assays clinical, rat, chick, microbiological). B_{12b} and B_{12} form two separate pink bands on silicic acid columns, using a concentrate from *Streptomyces aureofaciens* as starting material (Pierce *et al.*, 1949). B_{12b} has been crystallized in small red rods. It contains Co and P, is biologically active (chicks, *L. leichmannii*) and has a spectrum differing slightly from that of B_{12}. It has been recently reported to have the same potency as B_{12} in chick, microbiological and clinical assays (Stokstad *et al.*, 1949d).

Smith, who first reported on this chromatographic procedure, also obtained two separate pink bands using liver concentrates (Smith, 1948a; Smith and Parker, 1948); both fractions were clinically active, "one apparently resulting from the proteolysis of the other" since the faster moving one was prominent only in preparations of papain digested liver, not in preparations from undigested liver. Possibly one of these was B_{12b}. Winsten and Eigen (1949a) and Winsten *et al.* (1949) using paper strip chromatography have reported the presence of two zones, both active microbiologically, in commercially available crystalline B_{12} specimens. Their evidence indicates that these were not formed from one substance during the chromatography that lasted overnight, although Hendlin (Hendlin and Woodruff, 1949) stated that in similar experiments lasting 3 days, an originally homogeneous B_{12} is converted to an equilibrium mixture of B_{12} and B_{12a}, containing about 10% of the latter. (Statement not included in printed abstract.)

Smith first erroneously reported three P atoms per molecule of his red crystals (Smith, 1948b) indicating a different product from that of the Merck group and Ellis and coworkers, but this error was later corrected (Smith, 1949).

2. Concentrates

In the present state of knowledge the earlier observations on the behavior of B_{12} activity in crude concentrates are still of interest although the applicability of these properties to pure B_{12} must be regarded as questionable. First of all it is obvious that impurities present may affect the behavior of the activity. Secondly, the activity in crude sources may actually be present largely in various conjugated forms. The process of preparing pure B_{12} has not been described by anyone. Smith found it necessary to digest his concentrates with trypsin (Smith and Parker, 1948) or "mixed bacterial enzymes" (Smith, 1948a) to get final purification. It is not clear whether this procedure was effective simply by changing the adsorption and other properties of accompanying impurities, or whether the compound(s) carrying the activity were also altered by the removal of a peptide portion not necessary for activity. If the latter is the case the active material in natural sources may have quite different properties from pure B_{12}. Keeping in mind the reservation that the properties of the activity in crude materials or concentrates may be referable to larger molecules, conjugates of B_{12}, we will briefly summarize what properties have been consistently reported for what appears to be B_{12} activity in liver and fish solubles and the digest of Bird's acid precipitate from cow manure. These properties should continue to be of interest as long as there is a point in fractionating liver to concentrate other biologically active compounds.

The earlier work on concentrating anti-pernicious anemia activity has been reviewed by SubbaRow *et al.* (1945). The papers which are most informative on the properties of the primary liver factor (B_{12} active materials) are those of Cohn *et al.* (1928), Dakin *et al.* (1935, 1936), Karrer (1941), Kyer (1935), Sladek and Kyer (1938), Laland and Klem (1936). Of the recent papers, only Smith (1948a) describes any properties of liver concentrates. In the work on concentration of B_{12} active materials tested on laboratory animals, the papers of Bird *et al.* (1948), Jaffe and Elvehjem (1947), Pensack *et al.* (1949), Robblee *et al.* (1948a) and Zucker and Zucker (1948a) contain pertinent data. In addition, we have drawn upon a few unpublished results.

Solubilities—the active material is soluble in water, glycerine, glacial acetic acid, 90% phenol, and insoluble in ether, petroleum ether, chloroform, anhydrous acetone and anhydrous pyridine. It is soluble in aqueous ethanol and aqueous acetone. Somewhat more water is needed for a given degree of solubility with acetone than with ethanol. *Liquid-liquid distribution*—phenol-water: the activity is all in the phenol, but the addition of ether shifts the activity to the aqueous phase. *n*-BuOH-water: the activity is largely if not entirely in the aqueous phase, but the distribution ratio is shifted somewhat in favor of the butanol phase by the addition of phenol or ammonium sulfate. Aqueous acetone-saturated aqueous ammonium sulfate: activity mostly in acetone phase. *Salting out*—one-third to full saturation of aqueous solution with ammonium sulfate precipitates activity, but incompletely; precipitation at pH 3 gives better recovery. *Precipitating reagents*—the activity is not precipitated by Ca^{++}, La^{+++}, Ba^{++}, picric acid, trichloracetic acid, or basic lead acetate at a pH below 7.5. It is precipitated by phosphotungstic acid at pH 3 or below and also by Reinecke salt. These last reagents still give precipitates with very potent concentrates of B_{12}, but it was not stated whether the activity is precipitated (Smith, 1948a). *Adsorption*—the activity can be adsorbed on charcoal from aqueous solution. Neutral or slightly acid solutions have usually been used. Some failure to adsorb, or incomplete adsorption has been reported for animal protein factor activity (McGinnis *et al.*, 1947b; Bird *et al.*, 1948). Obviously unless a substance in a given solvent has a very favorable adsorption isotherm, it will be distributed between solution and adsorbate during either adsorption or elution procedure when one or at most two equilibrations are made. Then, depending on the concentration of the substance or substances to be adsorbed and the concentration of the charcoal suspension any result can be obtained from pretty complete adsorption to little adsorption. In our hands fairly complete adsorption of all activity from crude concentrates requires large amounts of charcoal; thus starting with 25 g. of a crude concentrate with activity corresponding to 4 mg. B_{12}, in 750 ml. volume, 25 g. Norit adsorbed 25% of the activity, and 120 g. was required to adsorb 90% of the activity. It cannot be said at this time whether this behavior is directly that of B_{12}, of a B_{12} conjugate, of a series of B_{12} active substances of differing adsorption properties, or to what extent the results are affected by impurities present which have displacing properties. Ninety percent phenol is an extremely efficient eluting agent; hot 50 or 65% ethanol is less efficient but more selective. *Chromatography*—Smith and Parker (1948) describe the use of alumina columns with water as solvent to remove dark impurities, and the use of columns of wet silicic acid or dry starch, with aqueous *n*-butanol, *n*-propanol or isopropanol with or without dissolved phenol to adsorb and purify the active material. Winsten and Eigen (1949a) and Winsten *et al.* (1949) describe paper strip chromatography using *n*-butanol saturated with water, which separates B_{12}-like factors from desoxyribosides, and the same solvent containing glacial acetic acid, which separates 2 or 3 B_{12}-like factors (B_{12}, B_{12a}, B_{12b}?) from each other.

3. *Terminology*

The term *animal protein factor* (APF) is generally applied to the factor or factors necessary for the maintenance of normal hatchability and growth of young chicks subsisting on a practical ration which does not contain a crude animal protein supplement. Subsequent usage has branched out in several directions; the term has been applied to a factor needed by mice on a purified casein diet (Bosshardt *et al.*, 1949c) by rats on cottonseed or soybean meal diets (Zucker and Zucker, 1948b; Van Landingham and Lyon, 1947), by *Lactobacillus casei* (Daniel *et al.*, 1948). It has now been shown that a deficiency common to all these conditions (except possibly the last) is supplied by crystalline vitamin B_{12} which is certainly therefore the principal animal protein factor.

The term *vitamin* B_{12} is apparently being used by its originators for a single chemical entity of defined crystalline form, solubilities, spectral absorption, etc. Closely related compounds sharing the same kind of biological activity are designated as B_{12} with a subscript—B_{12a}, B_{12b}, etc. We have tried to use the term B_{12} *activity* for that biological activity which can be obtained in higher animals from B_{12}. Then B_{12a}, B_{12b} and possibly still other relatives and conjugates thereof show B_{12} activity, although not necessarily the same amount of activity per molecule or the same relative activity for different species.

There is currently an attempt to restrict the term animal protein factor to a chick growth factor demonstrated by use of rations based on soybean meal and corn which have been typically used in much of the chicken work. The term *animal protein factor complex* has been used by Stokstad *et al.* (1949c) for a group of factors needed on such a diet. Their experiments indicate that crystalline B_{12} does only part of the job. APF complex might then be understood to include the B_{12} family which provide B_{12} activity, plus sources of one or more other biological activities, all of them needed by poultry on a corn-soy ration.

The terms *cow manure factor* (Rubin and Bird, 1946a, b; Bird *et al.*, 1948), *X factor* (Hartman, 1946), *zoöpherin* (Zucker and Zucker, 1948a), *Lactobacillus lactis Dorner* or *LLD factor* (Shorb, 1948), *L. leichmannii factor* (Skeggs *et al.*, 1948) and *extrinsic factor* (Castle *et al.*, 1944) are used for activities as tested by particular procedures in all of which crystalline B_{12} is effective. The alternate technique with *L. leichmannii* (Stokstad *et al.*, 1949a) using thioglycolic acid in the medium may lead to another subdivision in terminology. (see Section V, 1). A number of names applied to unknown factors in the recent literature are listed in Table I, classified according to whether they represent principally B_{12} activity, pteroylglutamic acid activity, or unknown activity. Some of these are discussed in more detail in Section VII.

TABLE I

I. Names which have been applied to factors whose activity is wholly or in part ascribable to B_{12} activity

Animal protein factor (APF)	Term probably originated at Cornell
Hatchability factor	Bethke *et al.*, 1946
Cow manure factor	Rubin and Bird, 1946a
Zoopherin	Zucker and Zucker, 1948a
Nutritional factor X	Hartman *et al.*, 1949b
Antithyroid factor*	Emerson, 1949
Antipernicious anemia factor	West, 1948
L. lactis Dorner factor (LLD)	Shorb, 1948
L. leichmannii factor	Skeggs *et al.*, 1948

II. Names recently applied to factors whose activity is not ascribable to B_{12}

A. When active for a given species the activity is that of pteroylglutamic acid
Folic acid, Vitamin B_c, Vitamin B_c conjugate, fermentation folic acid, factor R, Vitamin B_{10}, Vitamin B_{11}

B. Unknown factors (some may be identical with each other; some might be known factors)

		Sources
Monkey factor	Ruegamer *et al.*, 1948b	Whole raw liver
Antithyroid factor*	Ershoff, 1949b	Whole liver
Factor S	Schumacher *et al.*, 1940 Charkey *et al.*, 1947	Yeast
Carlson factor	Carlson *et al.*, 1949	
Vitamin T	Goetsch, 1946a	
Vitamin B_T	Fraenkel, 1949	
Vitamin B_{13}	Novak and Hauge, 1948	Distillers' solubles
Unknown APF factor	Stokstad *et al.*, 1949d	*Streptomyces aureofaciens*
Tomato juice factor (TJ)	Shorb, 1947	Tomato juice
L. citrovorum factor	Sauberlich and Baumann, 1948	Liver
Protogen	Stokstad *et al.*, 1949b	Liver

* Under certain conditions the deficiency induced by feeding thyroid is a B_{12} deficiency; under other conditions the deficiency does not respond at all to B_{12}.

III. Animal Work with "All-Plant" Rations

1. *The Deficiency State in Chicks and Rats*

If an all-plant ration (typically based on soybean meal and cereal grains) is fed to laying hens that do not have access to their droppings, hatchability of the eggs falls to low levels, embryos dying shortly before hatching. There is also high mortality of newly hatched chicks, with slow growth of the survivors, which carries over into delayed maturity (Wilgus and Zander, 1944; Bird *et al.*, 1946). These phenomena of low hatchability and poor growth of the offspring do not seem to be associated with any decline in egg production or loss of body weight by the hens (Wilgus and Zander, 1944; Bethke *et al.*, 1946; Cravens *et al.*, 1946; Parkhurst and Kuzmeski, 1946).

If a deficient all-plant ration is fed to breeding rats there is considerable mortality of the weanling offspring, with slow growth of the survivors (Zucker and Zucker, 1948a). These phenomena are not necessarily associated with any reduction in quantity of milk secreted, judging by the normal growth and survival of the young in the first 2 weeks after birth, or with loss of body weight by the mothers (Zucker and Zucker, 1948d). As in the chicks there is no characteristic pathology in the young rats which can be directly attributed to the deficiency.

In curative experiments the young, both chicks and rats, respond with rapid growth and much reduced mortality to an effective source of the factor. In the rat, preventive experiments involving the addition of crude casein, fish solubles or liver extract led to reproduction, lactation and growth equalling that on good stock diets (Zucker and Zucker, 1948a).

2. Characteristics of the Deficient Diets

Much of the work on all-plant rations has been done with so-called "practical" rations—i.e., diets which for reasons of cost and availability of materials are kept as simple and free of extra supplements as possible. Consequently, many of them are characterized by multiple mild deficiencies. Phillips and collaborators working with rats started out with a diet deficient in a great variety of factors, and many of the detailed findings reported have since yielded to supplements of known factors (Marüyama and Phillips, 1948). A somewhat better type of diet in terms of known factors has been used in much of the poultry work, and in rats by Van Landingham and Lyon (1947). The composition is typically 20–35% soybean meal, a simple mineral supplement, a source of vitamins A and D, riboflavin or a riboflavin concentrate, a little alfalfa leaf meal, and cereal grains to 100%. Hatchability is apparently not improved by adding a variety of known factors to this type of diet (Bethke *et al.*, 1947a). Many investigators, however, have reported improved growth of offspring with additions of methionine or choline, pantothenic acid, niacin and biotin, although usually finding the response to an active supplement of animal protein too great to explain on the basis of these known factors (see, e.g., Rubin and Bird, 1946a; Bird *et al.*, 1947). In more recent work (Ott *et al.*, 1948; Ott, 1949; Robblee *et al.*, 1948a, b; Stokstad *et al.*, 1949c) diets have been thoroughly supplemented with all available known factors except B_{12} without suppressing the need for B_{12}. The list of added factors in these diets includes thiamine, riboflavin, pyridoxine, pantothenate, niacin, choline, biotin, pteroylglutamic acid, alpha tocopherol, a source of vitamins A, D and K activity, besides sometimes inositol, *p*-aminobenzoic acid and methionine.[2]

The work on rats leading to the findings reported in the preceding section was done with a diet based on a nontoxic cottonseed meal, and containing yeast and alfalfa to supply some known and unknown factors, as well as added thiamine, riboflavin, pyridoxine, pantothenate, niacin, choline, alpha tocopherol, and sources of vitamins A, D and K (Zucker and Zucker, 1948a). It should be noted that this diet has an unusually high protein content (somewhat over 40%), and it is not clear whether some of the findings reported may be affected by the protein level. This also applies to diets of other investigators, e.g., most of the diets more recently used in chicken work. None of the findings in the rat work cited are referable to choline deficiency (a point raised by the work of Schaefer *et al.*, 1949a), since the cottonseed meal supplies considerable labile methyl, the diet contains an additional 0.3% choline, and the omission of this extra choline had no effect in exacerbating any of the symptoms.

3. Other Relevant Conditions as They Apply to All-plant Rations

a. Maternal Carry-Over and Storage. A common finding with chicks, rats and mice is the ability of a well-fortified maternal diet to protect the offspring placed on a deficient diet from any growth inhibition for several weeks after hatching or weaning. (Bethke *et al.*, 1947b; Bosshardt *et al.*, 1949c; McGinnis and Carver, 1947; Rubin and Bird, 1946b; Zucker and Zucker, 1948a.) These observations suggest that rat's milk produced on deficient diets must be low in the protective factor, and that eggs laid on deficient diets must be similarly low. It has been found that such eggs are deficient (Rubin and Bird, 1947), and that injecting crystalline B_{12} into deficient eggs can protect the chicks (Lillie *et al.*, 1949).

Supposedly normal chicks from commercial hatcheries have usually (as of 1947) shown depressed growth in experiments of a few weeks duration on the typical soy-corn ration, and have responded to crude animal protein supplements. This was so, provided they did not have access to range or to their droppings. Variability among different lots of chicks in this respect has, however, been great, and the results are quite consistent with the idea that the management of commercial breeding flocks was such as to supply a suboptimal amount of the factor, allowing acceptable hatchability without enough carry-over to the chicks for protection. How much the recent new knowledge has already changed practices is hard to tell; current experimental work is being done with chicks from hens fed an experimental diet of known deficiency.

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[2] We specifically list the factors because the exact meaning of the frequently used phrase "all known factors" depends on the year or even the month of publication.

Four sets of observations on the performance of supposedly normal weanling rats (from stock colony) on all-plant rations are available. In three cases early growth was called normal (Zucker and Zucker, 1948a; Van Landingham and Lyon, 1947; Schaefer *et al.*, 1949a, the rats on the choline supplemented diets); in one case it was depressed slightly (Jaffe and Elvehjem, 1947; Sporn *et al.*, 1947). By comparing the two papers of the Alabama group (Schaefer *et al.*. 1949a, b) it can be seen also that on essentially the same diet (crude plant diet supplemented with choline but with no source of B_{12}) stock rats showed early normal growth with no response to B_{12}, but chicks grew poorly and required a B_{12} supplement. Breeding diets for rats may be generally better supplemented in relation to the requirement than breeding diets for poultry have been, or the rat with a given degree of dietary deficiency may be better able to protect its offspring than is the hen.

b. Intestinal and Fecal Synthesis. The fresh droppings of the hen maintained on a deficient diet are not a significant source of animal protein factor activity, but droppings left at 30°C., for 72 hours are an excellent source (McGinnis *et al.*, 1947a), as are feces collected and dried without special precautions taken to prevent further microbiological activity (Rubin *et al.*, 1946). Birds with access to their droppings, and especially birds kept on built-up litter changed infrequently show little or no requirement for a dietary source of the factor (Kennard and Chamberlin, 1948, Kennard *et al.*, 1948).

No intestinal or fecal synthesis has been established in rats on all-plant rations. Feces were collected from lactating females on a quite successful stock diet and on an all-plant ration, and prepared for testing under conditions that would allow considerable additional microbiological activity; the stock feces were highly active for rat growth at 10% in the test diet, whereas the deficient feces were inactive. In some experiments the deficient feces were air-dried at room temperature and mixed in the diet at 10%; in others freshly collected undried feces were separately fed *ad lib* to the test rats, with a record kept of the amount consumed (Zucker and Zucker, 1950). It is not known how much of the activity of the stock feces came from the food and how much from microbiological synthesis. It may be noted that the deficient diet used in this work had little available carbohydrate, except for 15% glucose; the cottonseed meal which constituted the bulk of the diet contained only traces of starch and large amounts of raffinose (Dollear and Markley, 1948) which is not available to many organisms. The chicken work with fecal synthesis was done with diets containing considerable amounts of starch from grains.

c. Protein Level. The deficiency is much exacerbated, and the requirement for the missing factor is much increased (Heuser and Norris, 1944; Zucker and Zucker, 1948a; Bird *et al.*, 1948; Stokstad *et al.*, 1949c), by raising the level of seed meal in the ration. Levels of 60 or 70% are now commonly used to get severe deficiencies.

4. Effectiveness of Crystalline B_{12}

a. On Natural Diets. Most experiments with crystalline vitamin B_{12} so far reported involved short-time curative responses in depleted animals. B_{12} has been found to give a good response in chicks on a well-fortified soy diet (Ott *et al.*, 1948; Ott, 1949); in rats from depleted mothers on a well fortified diet (Emerson, 1948; Emerson *et al.*, 1949a, b) in chicks from depleted or undepleted hens on a practical corn-soy ration (Lillie *et al.*, 1948); in rats from depleted mothers on a well-fortified cottonseed-yeast-alfalfa diet (Zucker and Zucker, 1950); in chicks on a well-fortified soy-corn ration (Stokstad *et al.*, 1949c). In addition pure B_{12} rapidly restores hatchability to approximately normal levels on an all-plant ration (Carlson *et al.*, 1949; data not in printed abstract). Findings on pigs fed typical all-plant rations are somewhat contradictory. Catron *et al.* (1949) using a practical corn-soy-alfalfa ration recorded that B_{12} was an effective supplement. Cunha *et al.* (1949) with a corn-peanut meal ration partially supplemented with known factors got no effect from B_{12}, although growth was stimulated markedly with a crude animal protein factor concentrate of microbiological origin which contains other things besides B_{12} activity. It is possible that there is an unknown deficiency other than that of B_{12} activity in Cunha's diet; however, peanut meal is low in methionine and it is not clear whether this is entirely remedied in the pig by the amount of choline in the diet.

As a preventive study with crystalline B_{12} on a crude all-plant ration there is so far only the work of Emerson *et al.* (1949a, b). Breeding rats and their offspring on a 60% soy diet fully supplemented with known factors received liberal amounts of B_{12}. The weights at 90 days postweaning were 236 g. for these rats and 248 g. for suitably matched stock controls. It is to be hoped that evidence will soon be available to show whether optimal or near optimal performance over several generations is attainable by B_{12} supplementation of this type of diet, or on diets containing further supplements of unknown factors from natural sources which are free of B_{12}.

Comparison in short-time curative experiments of the ability of pure B_{12} to replace an equal amount of B_{12} activity from crude sources is of dubious value because of the shortcomings of the B_{12} assay of crude materials. A more valid experimental procedure at this stage for investigating whether other factors are needed is to determine whether the maximal effect possible with large doses of B_{12} equals or does not equal the maximal effect possible with crude sources. The maximal response to B_{12} was stated to be as good as the maximal response to liver or fish preparations by Ott (1949), Nichol *et al.* (1949a), Zucker and Zucker (1950). Stokstad *et al.* (1949c) on the other hand found that maximally effective levels of B_{12} (up to 2.5 gamma per 100 g. of diet) in chicks fed a well-fortified soy diet gave only half the growth that was obtained with a crude APF preparation from *Streptomyces aureofaciens.*

b. With Thyroid-Active Substances. The addition of a thyroid-active substance to various plant rations which should be deficient in animal protein factor is reported to increase the growth deficit in chicks from deficient hens (Robblee *et al.*, 1948c, using iodinated casein) and in stock rats (Emerson, 1949, using desiccated thyroid, and Register *et al.*, 1949b, using iodinated casein). It has long been established that feeding such material increases the requirement for various nutritional factors—specifically thiamine, pyridoxine, and pantothenic acid (Drill and Overman, 1942). An increased need for animal protein factor may be another example of a very general phenomenon. This very fact raises difficulties in evaluating results obtained by thyroid feeding; not only must all the known factors be given in sufficiently large amount, but apparently response to other substances not considered nutritional essentials can turn up (see Section VII, 2). The question may be raised whether in such experiments the iodine content of the diet should not be given consideration and whether, in the case of soybean meal diets the goitrogenic effect of this component can be disregarded. That rats on such diets respond to crystalline vitamin B_{12} is shown by Emerson (1949) and Register *et al.* (1949a). Emerson's work indicates that animals on the basal ration without thyroid are underweight after 6 weeks, and that after 2 weeks of B_{12} treatment the thyroid fed animals have not reached the weights of the animals on the basal ration without thyroid.

5. Summary

General conclusions to be drawn from the work with all-plant rations are: (1) Deficiency of B_{12} has been reliably and reproducibly produced on such diets in several species and a great many different laboratories; (2) Whatever other deficiencies there are in such diets, B_{12} is a limiting factor of importance on growth and performance, so that a response to

B_{12} can be generally counted on (exception, Cunha *et al.*, 1948); (3) Indications of another deficiency not supplied by B_{12} activity are variable and may be large (Stokstad *et al.*, 1949c; Cunha *et al.*,(?) 1948) or vanishingly small (Zucker and Zucker, 1948a, 1950).

IV. ANIMAL WORK ON "PURIFIED" RATIONS[3]

1. *General Considerations*

To facilitate the discussion it may be well to consider the following. After the isolation of pantothenic acid it became possible to maintain rats and mice for several generations on a purified casein diet with pure B factors—thiamine, riboflavin, pyridoxine, pantothenic acid, niacin, and choline. Lactation and growth were consistently found to be inferior (Foster *et al.*, 1943; Henderson *et al.*, 1942; Jukes, 1940; Unna *et al.*, 1941; Vinson and Cerecedo, 1944). With the chick, even so much of an achievement as this had to wait until biotin and folic acid were available, as survival, egg production, and hatchability are too poor with these deficiencies. The literature dealing with the growth of chicks on purified diets before pteroylglutamic acid (PGA) was readily available is in a very confused state; it must be borne in mind that various crude materials and concentrates which were supposed to be supplying still other factors thought to be required by the chick—factor R, vitamins B_{10} and B_{11}—were actually simply supplying folic acid activity in a form which did not register as such in microbiological assays.

On a diet containing biotin and PGA in addition to the factors used in the rat studies, and, like the rat diets, containing no added B_{12}, chickens have been maintained for several generations but with very inferior reproduction and growth (Jukes *et al.*, 1948). The addition of these two factors to the diet of the rat or mouse has somewhat improved preweaning growth and survival, and weight maintenance in the lactating female (Bowland *et al.*, 1948; Henderson *et al.*, 1948; Nelson and Evans, 1947; Mirone and Cerecedo, 1947; Sica *et al.*, 1948; Sporn *et al.*, 1947; Williamson, 1949) or had no effect (Folley *et al.*, 1947). Studies with sulfa drugs have established the important role of folic acid in growth, reproduction and lactation in the rat, but intestinal synthesis and coprophagy may normally supply the rat with a very considerable intake, apparently actually an optimal intake in the case of Folley *et al.* However, the general performance of rats and mice on fully supplemented diets without B_{12} remains suboptimal. The indicated *degree* of deficiency of purified diets for rats containing all the known factors except B_{12} is quite different in different laboratories. Classifying results in a roughly graded series, we find very severe deficiencies reported by Cary and coworkers (Cary *et al.*, 1946; Cary and Hartman, 1947; Hartman *et al.*, 1949b) and by Bowland *et al.* (1948), moderate deficiencies by Folley *et al.* (1947) and Schultze (1949), and mild deficiencies by the others mentioned above. Of these the severe deficiency found by Cary *et al.* responds well to administration of B_{12} but evidence is not available that the response is maximal. In several other cases (Folley, Sporn) usually reliable B_{12} sources do not markedly or reproducibly remedy the situation. Other evidence which will be discussed fully in Section VII, 2, strongly indicates the existence of still unknown factors, some of which are probably automatically supplied by the crude constituents of the all-plant rations.

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[3] As stated, the nutritional phase of B_{12} studies had two origins: with practical chick rations and with casein diets. For the class to which the former belong we can use the terms "crude rations," "natural rations" or "all-plant rations" and they convey a fairly definite meaning. The latter type which with hesitation we designate here as "purified rations" has no name now in use which is also a reasonably accurate description. Casein diets and alpha-protein diets can quite properly be thus designated, but some investigators have also used the term rather broadly for e.g., a ration largely made up of soybean meal, apparently because pure vitamin sources were used. The degree of purification of casein might also make a difference in the applicability of the word "purified." For present purposes the important character of the "purified diet" is that a separated or isolated protein source was used which had been freed of B_{12} activity or was originally so.

2. Other Relevant Conditions As They Apply to "Purified" Diets

a. Maternal Diet. Where it has been shown that B_{12} is a prominent deficiency in a purified or semi-purified diet, the findings share many of the features characteristic of the work on all-plant rations. Thus, there is a strong effect of the maternal diet (Cary and Hartman, 1947); also the offspring of mothers on all-plant rations have been used for experimental work with purified diets (see Section IV, 3).

b. Protein Level. It has been thoroughly demonstrated that the severity of the deficiency steadily increases as the casein level is raised (Cary *et al.*, 1946; Cary and Hartman, 1947; Bosshardt *et al.*, 1949b). In Cary's work there was 100% mortality within 2 weeks after weaning of depleted rats raised on 65% casein. In Bosshardt's work there is enough protection from the maternal diet (stock) so that mice grew well on a diet with 20 or 30% casein, but required crystalline B_{12} for equal growth on 45% casein.

c. Purity of the Protein Source. Most purified diets have relied on casein for the protein source. Taking together the results of the early Euglena work of Hutner (1936), the assay for extrinsic factor (Castle *et al.*, 1944) and the animal work (Cary and Hartman, 1947; Cary *et al.*, 1946; Hartman *et al.*, 1941; Zucker and Zucker, 1948a), it would appear that crude casein can carry appreciable B_{12} activity, and that either repeated isoelectric precipitation or repeated extraction with ethanol (preferably hot) yields caseins at least very low in B_{12}. Cary *et al.* have consistently purified their casein by exhaustively extracting with hot alcohol, and report a very severe deficiency in rats which responds to B_{12}. However, B_{12} deficiencies have also been successfully produced in chicks, pigs, and mice on either Labco vitamin "free" casein or GBI vitamin test casein (see Section IV, 3) and the Labco product has been found free of extrinsic factor activity at 50 g. per day (Castle *et al.*, 1944). These are the two commercially purified caseins commonly used on this continent. The purified casein used by Folley *et al.* (see also Mapson) was the British Drug Houses or Glaxo House product.

The recently available isolated purified protein from soy (Glidden Co.) should be free of B_{12} activity without further purification, but it may provide other unknowns carried by soybean meal. It is interesting to note that a two-generation experiment (Schultze, 1949) using a purified diet based on this protein did not apparently lead to as severe a growth depression in rats as that found in Cary's work.

d. Intestinal or Fecal Synthesis. In contrast to the negative findings for B_{12} activity in feces of rats on a crude plant ration based on 60% cottonseed meal (see Section III, 3 b), Hartman *et al.* (1949a) report positive findings on a "purified" ration when a liberal B factor allowance is included. The experiments were started at parturition. The post-weaning growth of the offspring was very variable; the best growing rats grew no better with liver extract, while the poorly growing rats responded to liver extract or to a small dose of feces from well growing rats. This response to the feces was suppressed if the rat eating the feces was on a diet containing succinylsulfathiazole. If the B complex was supplied at considerably lower levels the growth of the offspring was uniformly poor; then feeding of feces from well growing rats on the high B levels to the poorly growing rats on the low B levels was without effect. When B factors were singly reduced from the high to the low level, it is stated that riboflavin turned out to be the important factor. The riboflavin level inadequate for synthesis was about 71 μg. per day; information on the high level adequate for synthesis is not available. The low level might correspond roughly to 0.5 to 0.8 mg. per 100 g. of diet. Riboflavin levels used in purified diets in various laboratories range from 0.3 to 2.0 mg. per 100 g. The crude plant ration on which no synthesis could be detected contained 2 mg. per 100 g. as added riboflavin, plus the riboflavin of the yeast and alfalfa in the diet, plus that of the 60% cottonseed meal which is itself a good source.

Bosshardt *et al.* (1949a) have data indicating intestinal synthesis of B_{12} in mice. On a purified casein diet containing 2% succinylsulfathiazole, with adequate supplements of biotin and PGA, growth was well below that of mice on the sulfa-free diet. Intraperitoneal administration of a liver concentrate containing 1% B_{12} activity produced a large, although suboptimal, growth response.

On the other hand Nelson and Evans (1948a; riboflavin 1 mg. 100 g.) and Williamson (1949; riboflavin 1.5 mg./100 g.) studying the performance of lactating rats and their offspring on purified casein diets, were able to counteract the effect of succinylsulfathiazole in the diet completely by supplying sufficient PGA and biotin. Nelson and Evans found such a diet either with or without the sulfa drug not quite adequate for normal performance, but the sulfa drug did not make the original diet any worse, so if intestinal synthesis of B_{12} was occurring at all in these rats, the sulfa compound could not inhibit it. Derse *et al.* (1948) repeated Cary's procedure, using 10% yeast to supply the B complex. The poor postweaning growth was not affected by substituting dextrin for glucose, which they interpreted as evidence against intestinal synthesis. However, using 10% yeast as the sole source of the B complex, Cary and coworkers reported no obvious interference (referable to intestinal synthesis) with production of the deficiency. Cravens and Halpin (1949) fed a purified diet to laying hens and found a lowering of hatchability which was prevented by fish solubles and was somewhat delayed by substituting dextrin for glucose; this was interpreted as being due to greater intestinal synthesis.

3. *Effectiveness of Crystalline* B_{12}

a. On Purified Diets. Good responses to pure B_{12} have been recorded in the growth of chicks from hens depleted on plant rations (Ott *et al.*, 1948, Labco casein); in the growth and survival of rats from mothers depleted on purified diets (Hartman *et al.*, 1949b); in the growth of mice from mothers depleted on plant rations (Bosshardt *et al.*, 1949c, GBI casein) as well as from undepleted mothers (Bosshardt *et al.*, 1949b); in the growth of pigs, maternal diet not described (Hogan and Anderson, 1949, Labco casein; Johnson and Neumann, 1949 and Neumann *et al.*, 1948; Labco casein and alpha soy protein); in the growth of rats from undepleted mothers, the diet containing no methionine and no other source of labile methyl (Bennett, 1949; Stekol and Weiss, 1949); in the growth of chicks from hens depleted on plant rations (Williams *et al.*, 1949). In addition a good response to fish solubles which may well be acting as source of B_{12} was found by Robblee *et al.* (1948b, GBI casein), (growth of chicks from hens depleted on plant rations), and by Cravens and Halpin (1949, GBI casein), (hatchability of eggs laid on a purified diet). In none of these cases is there as yet satisfactory evidence that

B_{12} alone restores normal lifetime performance—i.e., that there is not a residual deficiency of some other unknown factor.

Two instances of failure to get any response to B_{12} on a purified diet are the work of Bustad *et al.* (1948, GBI casein) and the work reported by Carlson *et al.* (1949, using alpha soy protein). There is also a variety of other evidence for additional unknown factors all of which will be considered in detail in Section VII, 2, which deals in unified fashion with all the evidence from whatever type of diet.

b. With Thyroid-Active Substances. Feeding of thyroid active substances on purified diets always depresses growth but may or may not lead to B_{12} deficiency. Ershoff found none (1949b). Betheil *et al.* (1949) found that pure B_{12} gave only a half-maximal response. Bosshardt *et al.* (1949c) found that high B_{12} concentrates were effective.

4. Summary

Work with purified diets presents more difficulties and problems of a different kind than work with crude plant rations. The extent to which casein has to be purified is still in doubt, particularly considering work with various species. Intestinal synthesis apparently comes into play more on purified than crude diets. There is a greater danger of encountering other unknown deficiencies on a purified diet than on crude plant rations. It is apparently more difficult to obtain B_{12} deficiencies in rats on purified diets than on crude diets and it is also more difficult to get B_{12} deficiencies on purified diets in rats than it is in chickens.

V. MICROBIOLOGICAL WORK

1. Testing

A microbiological technique using *Lactobacillus lactis* Dorner (Shorb, 1947, 1948; Shorb and Briggs, 1948) served in the isolation of crystalline B_{12} as carried out in the Merck laboratories (Rickes *et al.*, 1948a). Other lactobacilli which have been used for B_{12} or APF assay are *L. leichmannii* (Skeggs *et al.*, 1948; Hoffman *et al.*, 1948) and *L. casei* (Peeler *et al.*, 1949). Hendlin *et al.* (1949) have reported on different strains of *L. lactis* (6a and ATCC 8000). As reflected by the reports and discussion at a recent symposium on B_{12} (Am. Chem. Soc., 116th meeting, September 1949) there is no general agreement on a method specific enough to serve for industrial and pharmaceutical control purposes or for discriminating biochemical studies. This illustrates again that assay methods too crude for other purposes may be quite useful in isolation work if skillfully applied, decisions very often simply depending on which of two alternative fractions appears to be more potent.

The report of Shive *et al.* (1948b) that addition of ascorbic acid or even of water affected the assay outcome materially was quickly confirmed by others (Skeggs *et al.*, 1948; Greeve *et al.*, 1949). Partial clarification came from the observations of Welch and Wilson (1949) that a lower redox potential in the medium led to activation of certain B_{12} derivatives otherwise inert. The situation with regard to both the effect of dissolved oxygen in water and the action of reducing agents and other matters was much clarified by recent papers from the Merck laboratories (Koditschek *et al.*, 1949; Caswell *et al.*, 1949; Hendlin *et al.*, 1949). A favorable effect of adding thioglycolic acid to the medium has been discussed by Stokstad *et al.* (1949a).

The original work of Shive *et al.* (1948a) on the isolation of thymidine from liver extracts soon led to the finding that relatively large amounts of thymidine completely replace B_{12} for both *L. lactis* and *L. leichmannii* (Shive *et al.*, 1948b; Skeggs *et al.*, 1948). This action is apparently shared by other desoxyribosides, and to a more limited extent by desoxyribonucleic acid, but apparently not by desoxyribose. These facts emerge from a systematic study of 13 cultures of lactic acid bacteria as to their reactions to B_{12}, desoxyribosides and other new growth factors (Snell *et al.*, 1948, 1949; Kitay *et al.*, 1949).

Besides responding to B_{12}-active substances and desoxyribosides, the lactobacilli respond to a third category of substances whose chemical nature has not been reported. Their effect is made apparent by the "bioautographic" technique which combines paper strip chromatography with recognition of spots of activity on the strip by their ability to promote bacterial growth on an agar plate (Winsten and Eigen, 1949a, b; Winsten *et al.*, 1949).

Euglena gracilis is a chlorophyll containing flagellate whose nutritional requirements have been of interest for some time (see Hutner, 1936; Hall, 1943). It has recently been found to show growth response to crystalline B_{12} in concentrations about $\frac{1}{10}$ that required by *L. leichmannii*, but does not respond to thymidine (Hutner *et al.*, 1949). This specificity recommends it for assay purposes. However, it has been indicated recently (statement by Hendlin at B_{12} symposium, 116th meeting of the American Chemical Society, September 1949) that Euglena, like the lactobacilli, responds to several substances, apparently not desoxyribosides, which have no B_{12} activity in animal tests.

The conditions for more specific effects on microorganisms need to be worked out further both as regards substances which effect the B_{12} response *per se* and other unknown factors which can additively or synergistically affect the B_{12} results. Investigations in this field are very actively going on in many laboratories so that what is written now may be entirely out of date by the time this review appears in print. A reliable microbiological assay is so badly needed in biochemical work and for feed and pharmaceutical control that enough effort will be spent to achieve it.

2. Production

On various types of media, many microorganisms make B_{12} or B_{12}-active substances in large quantities. *Streptomyces griseus* (Rickes *et al.*, 1948c) and *S. aureofaciens* (Stokstad *et al.*, 1949c) are used in the commercial production of crystalline B_{12} and concentrates for the treatment of pernicious anemia, and APF concentrates for animal feeds. *Mycobacterium smegmatis*, *Lactobacillus arabinosus* (Rickes *et al.*, 1948c), *Bacillus subtilis* (Rickes *et al.*, 1948c; Stephenson *et al.*, 1948), *Bacillus megatherium* (McGinnis *et al.*, 1949), and *Aerobacter aerogenes* (Huff, 1949) are some of the known organisms which synthesize the activity. Organisms whose culture filtrates are not active include *Lactobacillus casei*, *Streptococcus faecalis* R and *Escherichia coli* (Shorb and Briggs, 1948). Unidentified organisms from chicken feces (Stokstad *et al.*, 1948), from putrefying brewer's spent grains (Ansbacher and Hill, 1949) and from the rumen of sheep (Zucker and Zucker, 1948c) have been shown to be active synthetically. Sifted top soil from land not manured for several years is a good source (Stephenson *et al.*, 1948).

The potency in these microbiological sources may be quite high,

TABLE II

Potency of Microbiological Materials

	B_{12} equivalence* μg. per g. of solids		
Organism	Whole culture	Cell filtrate	Cells
Streptomyces aureofaciens	4–6	1	10–13
B. megatherium (culture 6–10 hrs. old)	0.4–0.8 (μg. per cc.)		
Strained sheep rumen contents	1.2	≤.04	4.8
Protozoal fraction			6.3
Bacterial fraction			2.1
Mixed culture of rumen bacteria grown in sterilized rumen juice			4.0

* The first two lines represent APF activity for chicks expressed as the B_{12} equivalent. The figures were reported by Stokstad and by McGinnis at the B_{12} symposium, American Chemical Society, September, 1949. It should be noted that the particular assay used for the data of the first line responds only partially to crystalline B_{12} (Stokstad *et al.*, 1949c); therefore it is not clear how much of the B_{12} equivalence for the streptomyces represents B_{12} activity—i.e., could be supplied by crystalline B_{12}. There is much less room, if any, in the rat assay used for the sheep rumen fractions for activity other than B_{12} activity (Zucker and Zucker, 1948a, e; 1950).

higher than in whole liver and far higher than in most other mammalian and avian tissues. Some available data are shown in Table II (see also Table III).

Various investigators have dealt with problems of optimal vitamin production by microorganisms and their distribution between medium and cells as reviewed by Van Lanen and Tanner (1948). Developments in B_{12} production will contribute many new data of considerable interest.

TABLE III

B_{12} Activity of Various Non-Microbiological Sources

All figures are B_{12} equivalent, given as μg. per gram solids unless otherwise stated. Superscripts refer to notes at end of table which give method and investigator.

Liver products

Hog liver 1.8;[1] *calf liver* 2.4;[1] hot water soluble portion of hog liver (*Wilson* 1:20, Armour liver extract powder) 13.0, 7.3;[1] 6.0;[2] hot water insoluble liver residue (*Wilson A*) 0.3;[1] 70% EtOH insoluble portion of 1:20 liver (*Wilson S*, containing the bulk of the other B factors of liver including folic acid) 2.0;[1] 95% EtOH soluble portion of 1:20 liver (Wilson *liver paste*) 17 μg./g. (wet weight).[3] No figures available on Wilson liver L, an enzymatically solubilized preparation from Wilson S, or on the 70% EtOH soluble, 95% EtOH insoluble portion, the Cohn *fraction G*, which contains the bulk of the B_{12} activity of liver, probably at a considerably lower concentration than the liver paste. *Liver meal*, an animal feed which contains some liver and other organs, assayed at 0.55.[2]

Other meat products

Beef round 0.18;[1] 0.02 μg./g. fresh weight;[4] *calf spleen* 0.93;[1] *calf thymus* 0.24;[1] *pork shoulder* 0.01 μg./g. fresh weight;[4] *meat meal* 0.1.[2]

Fish products and lower forms

Fish meal 0.25,[2] 0.04;[5] *Fish solubles* 0.6–1.5 μg./g. wet weight,[1] 0.8–1.0 μg./g. wet weight,[2] 0.25–1.0 μg./g. wet weight;[5] *Crab meal* 0.33[5]; whole *starfish* 0.18;[1] *oyster* without shell 2.8;[1] eggs of *Artemia salina* (a small crustacean widely used for feeding tropical fish) 7.2;[1] *winkle* without shell 1.2;[1] whole *sand worm* 5;[1] whole *earth worm* 1.1.[1] In connection with earth worms, cf. top-soil, said by Stephenson *et al.*[6] to approach 1/10 the activity of liver paste.

Milk products

Milk 10–12 μg./qt.;[7] *crude casein* inactive to active, with one active preparation showing possibly as much as 1/50th the activity of 1:20 liver, i.e., 0.3;[1] *crude lactalbumen* (Borden 15–42), *purified caseins* (Labco vitamin "free," GBI vitamin test) ≤ 0.02.[1] Labco casein, and a casein extracted five times with 65% EtOH, cold, and once with 95% EtOH, hot, entirely inactive under conditions where 50 g. crude casein, 50 g. liver, 200–600 g. beef round were quite active.[8]

[1] Zoopherin activity (rat) Zucker and Zucker, 1948a, e, 1950.

[2] Cow manure factor activity, Lillie *et al;* data presented at the B_{12} symposium, American Chemical Society, September 1949.

[3] *L. leichmannii* activity, Gillis and Norris, 1949.

[4] Thyroid fed rat, Register *et al.*, 1949a.

[5] APF (chick), Ott, 1949. The fish meal was of poor quality (Ott, personal communication).

[6] APF (chick), Stephenson *et al.*, 1948.

[7] X factor activity (rat) Cary *et al.;* data presented at the B_{12} symposium, American Chemical Society, September 1949.

[8] Extrinsic factor activity (P.A. patient) Castle *et al.*, 1944.

VI. B_{12} REQUIREMENTS AND MODE OF ADMINISTRATION

The pernicious anemia patient, with his gastric and, possibly, absorptive disabilities, requires 30 or more times as much active material orally as intramuscularly, unless the material given orally is accompanied by the "intrinsic factor" of normal gastric juice (Minot and Strauss, 1943). Therefore it is of interest to compare modes of administration of B_{12} or concentrates in animals. As might be expected since the animal's digestive system remains intact, no large difference in favor of parenteral administration shows up. Bosshardt *et al.* (1949c), using mice on a purified diet containing iodinated casein, found it took about twice as much by mouth as intraperitoneally to give the same response. Stokstad *et al.* (1949c) reported it took 1⅓ to 2 times as much by mouth as intramuscularly in chicks. A similar or slightly larger differential might be inferred from the work of Nichol *et al.* (1947), where 0.1 cc. of a liver concentrate orally was not quite as effective as 0.05 cc. intramuscularly. It is not clear that any of these differences are significant. On the other hand Emerson (1949) obtained the same gains at several different levels of B_{12} given orally and subcutaneously to thyroid fed rats. In the authors' laboratory if there is any significant difference between parenteral and oral administration, the parenteral is more effective, but the dose ratio for a given response is certainly under 2. Such differences in experimental animals apparently have no bearing on the large difference established for pernicious anemia patients. Many drugs, where no such considerations as intrinsic factor enter, show a smaller activity when fed than when injected.

Some very approximate and preliminary data have been collected in Table IV. Consideration has been restricted to results with crystalline B_{12} and figures given are the doses required for a near maximal or maximal gain during a 2, 3, or 4 week test period. Practically all the results (exceptions, the pig data) are based on curative experiments. In making comparisons, the following facts should be considered. (1) Increasing the protein level, or (2) inducing a labile methyl deficiency, increases the requirement. It has been said that the chick requirement is doubled or tripled by going from a 30% soy diet to a 70%. (3) Feeding a thyroid active substance in the experimental diet, or (4) using a deficient maternal diet can induce a requirement apparent in short term experiments which would not otherwise be there. Little is known about the extent to which requirement increases further with increasing amounts of thyroid active material fed, or with increasing the deficiency or the depleting ability of the maternal diet. In view of these experimental uncertainties it is rather surprising that the figures for B_{12} in the diet cover the small range of 1.5–3 μg./100 g. of diet for depleted rats and chicks and undepleted pigs.

The data from our laboratory indicate essential agreement between the amount of B_{12} needed in the diet and the amount needed for separate administration, the rats eating about 250 g. during the 21 day test period.

TABLE IV

Dosage of Crystalline B_{12} for Approximately Maximal Response

Species	Crystalline B_{12}, μg.						
	Diet		In food	Separate			
	Maternal	Experimental	as μg. per 100 g. diet	Dose per day	Route	Schedule	Reference
Chick	Deficient	70 % Soybean	2–3				Ott, 1949
			2				Lillie *et al.*, 1948
			1.5*	0.04 0.06	I.M. Orally	Weekly Weekly	Stokstad *et al.*, 1949c
Chick	Deficient	28 % Soybean iodinated casein	1.5	0.1 or 0.5	I.M.	Every 2nd day	Nichol *et al.*, 1949a
Rat	Deficient	60 % Cottonseed	3	0.5 0.35	Orally I.P.	Daily Daily	Zucker and Zucker (1950)
Rat	Normal (stock)	60 % Soybean thyroid		0.125 0.125	Orally I.P.	Daily Daily	Emerson (1949)
		46 % Soybean iodinated casein		0.2	I.P.	Every 2nd day	Register *et al.*, (1949a)
		Purified casein thyroid		0.1–0.2		Daily?	Betheil and Lardy (1949)
		30 % Peanut (labile CH_3 deficient)	3				Schaefer *et al.*, (1949a)
		Amino acids; no labile CH_3, homocystine sole S amino acid		2–4	?	Daily?	Bennett, 1949
Pig	Normal	Alpha soy protein		2–5	I.P.	Daily?	Johnson and Neumann (1949)
		? % Soybean	1–2				Catron *et al.*, (1949)

* Stokstad *et al.* choose this figure. The data in their paper show almost as great an additional weight increment in going from 1.5 to 3 μg./100 g. diet as was obtained in going from 0 to 1.5.

On the other hand, Stokstad *et al.* have found separate administration very much more effective than administration in the diet; assuming a food intake of 100 g. per week, the required dose of B_{12} in the diet would be 1.5 μg. per week, as against 0.3 μg. per week if given separately. It should be noted that the separately administered B_{12} was given once a week by Stokstad *et al.*, daily by Zucker and Zucker.

It is stated in a recent publication (Reisner and West, 1949) that the pernicious anemia patient in relapse shows a maximal response with 1 μg./day (parenterally) while with 0.25 μg. there is a recognizable but sub-maximal response.

It is not clear from the paper of Hartman *et al.* (1949b) what is regarded as a just maximal dose of crystalline B_{12} for any of the diets reported. Data on the requirements of the mouse for crystalline B_{12} (Bosshardt *et al.* 1949b, c) are not as yet available.

VII. SOURCES AND DISTRIBUTION

1. B_{12} Activity

a. General Nature of Distribution. By general agreement liver (Gillis *et al.*, 1942; McGinnis *et al.*, 1944; Bethke *et al.*, 1946; Cravens *et al.*, 1946; Hartman, 1946; Heuser *et al.*, 1946; Cary and Hartman, 1947; McGinnis and Carver, 1947; Nichol *et al.*, 1947; Van Landingham and Lyon, 1947; Zucker and Zucker, 1948a), fish products, especially fish solubles (Heuser and Norris, 1944; Wilgus and Zander, 1944; Bethke *et al.*, 1946; Bird *et al.*, 1946; Cravens *et al.*, 1946; Heuser *et al.*, 1946; Lassen and Bacon, 1946; Bethke *et al.*, 1947a; Hill, 1948; Zucker and Zucker, 1948a; Nichol *et al.*, 1949a), cow manure (Rubin and Bird, 1946a; Robblee *et al.*, 1948a; Zucker and Zucker, 1948a), and cultures of a variety of microorganisms (see Section V, 2) are reliable and potent sources of B_{12} activity for animals. Meat products (Heuser and Norris, 1944; McGinnis *et al.*, 1944; Wilgus and Zander, 1944; Bethke *et al.*, 1946; Heuser *et al.*, 1946; Cary and Hartman, 1947) and milk products are apparently less potent or less reliable sources. The activity of milk goes with the casein (Cary *et al.*, 1946; Cary and Hartman, 1947; Zucker and Zucker, 1948a; Hartman *et al.*, 1949c). Some numerical results are set out in Table III. Certain materials are not considered useful sources because they constitute the bulk of diets which are deficient; these include cereal grains and other seeds (soy, cotton, peanut, pea and flax). Tomatoes and distillers' solubles (Hartman *et al.*, 1949; Zucker and Zucker, 1948a, 1950) as well as yeasts and alfalfa leaf meal must also be judged negative. For discussion and references on the last two materials see Section VII, 2.

b. Peculiarities of Distribution. No higher plant material has been found effective. In some of the chicken work with practical rations the addition of some known vitamin factors will lead to small improvement, and under these conditions some plant feeds also produce small improvements. But it appears that there is not one of the commonly available feeds of vegetable origin which can support even acceptable hatchability or good growth in chicks, and in well-fortified diets even the small supplementary effects disappear.

Such a distribution of a nutritional factor for animals is unprecedented. So far we have been able to rely on higher plant materials for a good supply of vitamins. All the known factors or their precursors occur among the plants that man and beast have chosen for their food. Many also are formed by yeasts and bacteria, but all such have recurred in higher plants. Even factors like ascorbic acid and vitamin E, which are not commonly found in these lower plant forms, are widely distributed among the higher plants. Williams (1943 and elsewhere) emphasizes the close association of the various B factors in a wide variety of species. It was this peculiar absence from higher plants and yeast, and presence in high concentration in rumen protozoa which led Zucker and Zucker to suggest the hypothesis that the factor was needed only by animals and played no significant role in plant physiology (Zucker and Zucker, 1948c). They were found to be regrettably in error as soon as it became known that molds and bacteria synthesize it in considerable amounts. *Euglena gracilis*, a borderline organism, containing chlorophyll but often classified among the protozoa, presents some interesting problems. It has been grown on inorganic media in the light (for discussion see Hall, 1943) but its growth is remarkably stimulated by B_{12} (Hutner *et al.*, 1949). In collaboration with Dr. Hutner a mass culture of *Euglena gracilis* was grown in the presence of 49 μg. of B_{12}, which yielded 25.4 g. of dry Euglena substance. Testing this material on rats showed that two-thirds or more of the total B_{12} was taken up and stored in the Euglena bodies (unpublished data). Thus, at least one kind of chlorophyll-containing cell utilizes and stores B_{12}.

If B_{12} has general physiological significance in higher plants as well as lower plants and animals, it should be possible to find some B_{12} somewhere in the plant. Examples of seeds, leaves, and fruits of higher plants having been found inactive, preliminary experiments have been carried out in the authors' laboratory on roots. The roots of beets and parsnips may contain just measurable activity as determined by rat assay (approximately .04 μg./g.; Zucker and Zucker, 1950). Cary and Hartman (1947) found carrots inactive. Soil has been found to be quite rich in B_{12} activity (Stephenson *et al.*, 1948). More data are needed on these points.

c. Available Assays. Data are given in Table III. It should be remembered that the Lactobacillus assays respond to a variety of substances, desoxyribosides and some unknowns which do not have B_{12} activity in animals. The clinical assay for extrinsic factor responds to PGA which does not affect the animal or microbiological assay (except as possible inhibitor in large doses). Some of the deficient chick diets, notably that used in testing for cow manure activity, respond partly to certain known factor supplements. The zoopherin assay (Zucker and Zucker, 1948a, 1950) shows no response to desoxyribonucleic acid or to any known factor including besides the known vitamins (PGA and others), choline, lysine, methionine, purified casein, and purified lactalbumin. The dose action curve for this assay (plot of response against log dose) is a straight line of constant slope for a variety of materials including crystalline B_{12}, whole liver, and microorganisms from the sheep's rumen. Similar investigations of the mouse assay using iodinated casein led to slopes for dose action curves which were not all the same. (Bosshardt *et al.*, 1949c.)

The zoopherin assay indicates that the hot water extract of whole liver (as represented by Wilson 1:20 liver and Armour liver extract powder) has about five times the potency of whole liver, with correspondingly low potency for the hot water insoluble fraction (Wilson A). These results are in agreement with the general assumption that the hot water extraction represents a step in concentration—this is also indicated by clinical assays (SubbaRow *et al.*, 1945). Furthermore, the zoopherin assay indicates that 1:20 liver is a reliable and stable source; about ten lots of 1:20 liver (Wilson), were used over a 3-year period as positive control in assays and also for fractionation procedures applied both to 1:20 liver and to fish solubles. One or other of the fractions from 1:20 liver being tested at levels corresponding to 0.25 or 0.5% original 1:20 liver showed without exception appreciable activity. Two samples of 1:20 liver powder which were assayed (2 years apart) gave about 55 and 80% response respectively at the above levels of 0.25% and 0.5% (unpublished data). Also zoopherin assays of four samples of whole liver gave approximately 75% response at 5% in the diet; these were Wilson dried hog liver, Viobin defatted 75° liver, fresh calf liver and fresh normal rat liver (Table III).

Bosshardt *et al.* (1949c) and Betheil *et al.* (1949) found whole liver and 1:20 liver of equal potency, and the latter group also alluded to the variability of 1:20 liver samples. Ershoff (1949b) whose animals do not respond to crystalline B_{12} at all but require whole liver, recorded responses varying from small to negative with 1:20 liver. Sporn *et al.* (1947) referred to the variability of 1:20 liver samples in supplying the activity provided by whole liver for growth and reproduction on purified diets. Our interpretation of these results is that 1:20 liver is a reliable and stable source of B_{12} activity, considerably more potent than whole liver. It is, however, an extremely variable and unreliable source of an unknown factor in whole liver. Any procedure which shows effects from 1:20 liver no greater than from whole liver, or very variable results from 1:20 liver, is one which may or may not represent a deficiency of B_{12} activity, but which certainly also represents a deficiency of another unknown liver factor. The importance of this point in the interpretation of observations in the literature is deemed sufficient to warrant the details given above.

Some results on the distribution of activity in animal tissues are presented in Table III. In addition Huff *et al.* (1948) reported the following activities of normal mouse tissue expressed in arbitrary units and based on *L. leichmannii* assays: spleen 5.3, pancreas 4.9, liver 4.0, lung 2.5, testes 2.3, heart 2.3, kidney 2.0, brain 1.7, muscle 1.0. The quantities of desoxyribosides in crude materials may be high enough to affect these assays (Lyman and Prescott, 1949a). In this connection it may be noted that the proportion of desoxyribonucleic acids in spleen is much higher than in liver (v. Euler and Hahn, 1948).

Livers of pernicious anemia patients coming to autopsy in relapse have no significant antipernicious anemia activity (Minot and Strauss, 1943). Livers of zoopherin-deficient rats tested for zoopherin activity showed ≤ 0.09 μg. per g. B_{12} equivalent, as compared with livers from rats on the same diet with a supplement of 5 or 10% fish solubles which assayed 1.3 μg. per g. (Zucker and Zucker, 1948b; Table III). Lewis *et al.* (1949) assayed various tissues obtained from rats on a deficient corn-soy diet and on the same diet plus 3 μg. B_{12}/100 g. diet. The assays were carried out on rats receiving a corn-soy diet plus thyroid. The tissues from the deficient rats were lower than those from the supplemented rats in all cases. The values for the tissues of the supplemented rats (apparently per gram moist tissue) are in μg. of B_{12} equivalent per g.: kidney 0.17, liver 0.08, heart 0.07, small intestine 0.04, spleen and leg muscle not measurable. The liver of a pig on a "purified" diet containing succinylsulfathiazole was found to be devoid of clinical activity (Welch *et al.*, 1948).

2. *Disputed Sources and Unknown Factors*

a. Alfalfa Leaf Meal. In several preliminary communications Cary and co-workers have reported lettuce, leafy feeds such as alfalfa leaf meal, and hays active under the same conditions where later crystalline B_{12} was found to be active (Hartman, 1946; Cary and Hartman, 1947). Zucker and Zucker (1948b) have reported failure to find appreciable activity in several samples of alfalfa or in fresh grass. Emerson *et al.* (1949a) found alfalfa inactive for rats, and McGinnis and Carver (1947) found a diet with 15% alfalfa ineffective in hens. Negative results of this sort on rats and chickens under conditions where B_{12} gives a response certainly establish that alfalfa is not a direct source of appreciable B_{12} activity. Possible explanations for the discrepancy are: (1) there is another deficiency in the Cary diet; (2) alfalfa stimulates intestinal synthesis of B_{12} activity in Cary's rats, as they state to be the case for riboflavin (Hartman *et al.*, 1949a), but is not effective in rats and poultry on plant rations; (3) there are traces of B_{12} in alfalfa, sufficient to register small effects under conditions where the B_{12} requirement is very small (low protein?). In this connection it has not been stated by Cary *et al.* how much of an effect is obtained with alfalfa, nor have the particular experimental procedures been published.

Other instances of alfalfa showing a favorable effect are the work of Bowland *et al.* (1948) studying reproduction on a purified diet, and Betheil *et al.* (1949) studying growth on a purified diet containing a thyroid active material. In the latter case at least it has been established that the procedure responds to something other than B_{12} activity.

b. Yeast. The unanimous agreement that yeast is not a source of B_{12} activity for animal experiments (Gillis *et al.*, 1942; Hartman, 1946; Patton *et al.*, 1946; Bethke *et al.*, 1947a; Cary and Hartman, 1947; Van Landingham and Lyon, 1947; Zucker and Zucker, 1948a; Hartman *et al.*, 1949a; Nichol *et al.*, 1949c) raises a question of interest, at least to those who teach, as to the name vitamin B_{12}. The name vitamin B complex has been used to cover the water soluble factors found in yeast. Vitamin C is a water soluble factor not found in yeast and has never been called a B factor. It is not of course absolutely excluded that yeast contains some B_{12} masked by the presence of a metabolic antagonist of B_{12}; nevertheless it must be realized that a diet which relies on yeast to supply the vitamin B complex will be deficient in vitamin B_{12} unless this factor is supplied from a source other than yeast.

Microbiological studies (Shorb, 1947) and clinical tests (Wintrobe, 1939; see also Strauss and Castle, 1932) show some activity in yeast. It is not clear whether the assays with Dorner or Leichmann organisms as now carried out in improved style still respond to yeast. Yeast of course contains some desoxyribonucleic acid (Chargaff and Zamenhof, 1948). For clinical effects very large doses of unautolyzed yeast are required (120 g. per day) and there are persistent indications that autolyzed yeast is much more effective than unautolyzed. The pernicious anemia patient will usually show a good hematologic remission, with all the signs used in testing for extrinsic factor activity, if he gets a good dose (1 mg. or more per day) of PGA (Darby, 1947). The response to conjugates—either the triglutamic acid (fermentation folic acid) or the heptaglutamic acid (vitamin B_c conjugate)—is slight or lacking. Folic acid activity in yeast is in the form of the heptaglutamate; however, it is not excluded that small amounts of the free PGA occur in yeast, especially in autolyzed yeast. It is also not excluded that some patients can respond to a large dose of the heptaglutamate.

The response to yeast by the thyroid-fed animals of Betheil *et al.* (1947) is presumably attributable to another deficiency in their diet.

A new chick factor occurring in yeast is indicated by the Cornell workers (Carlson *et al.*, 1949, data not given in printed abstract). Working with a purified diet containing 23.5% isolated soy protein plus amino acid supplements, with all the necessary known vitamin factors and minerals, they produced a deficiency in 3-week experiments with chicks which led to very poor growth, high mortality, some paralysis, and muscle hemorrhages. Crystalline B_{12} had no effect. Fully effective supplements were yeast and fish meal; partially effective were whey and liver paste. Since the deficiency does not appear on a diet containing soybean meal, the soybean must also contain the missing factor. Carlson suggested that it may be the same factor as that postulated by Hill (1948) who found that soybean meal was an extremely variable source while whey and fish products (fish meal, fish solubles) were good sources. The fish products gave better growth than the whey because they also supplied animal protein factor in the experiments of Hill.

In a series of earlier papers from Cornell, yeast was found to be necessary in various chick diets (Schumacher *et al.*, 1940; Gillis *et al.*, 1942; Hill *et al.*, 1944; Scott *et al.*, 1946a). The activity of yeast was ascribed to two factors—R and S; R has since been found to be folic acid conjugate (Charkey *et al.*, 1947), not needed in the presence of adequate PGA. Factor S has not been identified with a known factor, although it was suggested that it might be the strepogenin of Woolley (Sprince and Woolley, 1944; Woolley, 1946; Scott *et al.*, 1946b, 1947). "The role of strepogenin in the nutrition of the chick remains obscure" (Luckey *et al.*, 1947). At any rate, the new unknown factor needed on the purified soy protein diet may possibly be the same as Factor S.

Vitamin B_{13} is a factor recently postulated by Novak and Hauge (1948). It is needed for growth by chicks and rats. Its absence markedly affects the growth of undepleted stock weanling rats in experiments of 3 weeks duration. The activity is carried by casein, and the deficiency is produced on a purified diet using casein that has been thoroughly purified by a variety of procedures. Distillers' solubles, liver, and fish solubles are active sources. The chick experiments (Novak *et al.*, 1947) could be interpreted in the sense that distiller's solubles supplies only B_{13}, whereas fish solubles supplies B_{13} and B_{12}; a supplement of fish solubles therefore has a larger effect on growth than a good supplement of distillers' solubles. A high concentrate has been prepared from distillers' solubles; it is described as being a yellow-orange crystalline solid quite soluble in water, acetone, ethanol, ether, chloroform and benzene and the absorption spectrum shows a peak at 2820 A. (Novak and Hauge, 1948). Since distillers' solubles probably supplies everything that yeast supplies, and fish meal and fish solubles may well both supply the same factors, it is not ruled out that B_{13} and the Carlson factor and the Hill factor may turn out to be the same.

Two other postulated factors occurring in yeasts are needed for insect nutrition—Vitamin T (Goetsch, 1946a, b, 1947) and Vitamin B_T (Fraenkel, 1949; Fraenkel and Blewett, 1947)—the former needed for the development of the soldier termite, and the latter needed for the growth of *Tenebrio Molitor.* Yeast is an extremely rich source of vitamin B_T and B_T is said to be needed when all known factors including folic acid have been supplied. Vitamin T is found in penicillium and in the wild yeast *Torula utilis.* Neither factor is the same as B_{12}, because there is no B_{12} activity in either brewer's yeast or wild yeast (Zucker and Zucker, 1950).

c. Other Microbiologically Produced Factors. An unknown APF factor is postulated by Stokstad *et al.* (1949c, d). On a soy-corn diet chick growth gave only a partial response to crystalline B_{12}, while it responded fully to a crude APF preparation from *Streptomyces aureofaciens* cultures. The missing factor is absent from alfalfa and distillers' solubles, present in only marginal amounts in fish solubles and fish meal, present to an unspecified extent in the hot water extracted residue of beef and pork liver. In the experiments of Cunha *et al.* (1949), using a corn-peanut meal diet for pigs, a crude streptomyces preparation was active while B_{12} was not.

d. Cottonseed Meal. In comparing crude diets well supplied with all the known factors, including a source of folic acid, and based on either soy or on cottonseed meal for the protein source, Zucker and Zucker (1948d) found that while young rats produced on both diets showed the same principal deficiency (zoopherin, i.e., B_{12} activity), the early pre-weaning growth and survival of the soy offspring were inferior, and the mothers lost weight during their lactation period, whereas the cottonseed diet was normal in both these respects. This result was obtained with two non-toxic meals, a properly cooked hydraulic cottonseed meal and the Boatner gland-free meal. The moderate weight loss during lactation on a 60% soy diet has been confirmed by Emerson *et al.* (1949a, data not given in printed abstract). A difference between soy and cotton has also turned up when thyroid active material is fed (Ruegamer, 1949). Groschke *et al.* (1947) found that substitution of a properly detoxified cottonseed meal (the Boatner gland-free meal) for soybean meal in the

Bird deficient chick diet led to somewhat improved growth. It is possible that all these differences are due to the same unknown factor, not B_{12}, which is adequately supplied by 30% or more of cottonseed meal but not by 60% of soybean meal.

e. Whole Liver. Activity found in whole liver but not in B_{12} concentrates is needed probably in addition to B_{12} for growth and reproduction of rats on purified diets (Folley *et al.*, 1947), for growth of rats on purified diets fed thyroid active materials (Ershoff, 1947, 1949a, b; Betheil and Lardy, 1949), for growth and survival of monkeys, (Ruegamer *et al.*, 1948b) of foxes and of minks (Schaefer *et al.*, 1948a, b). In the work on monkeys, foxes and minks there is evidence that a part of the activity is very easily destroyed so that fresh raw liver is desirable or even necessary. A factor in raw liver seems to be needed by cats and not by dogs (Lockhart *et al.*, 1947). There may be several unidentified factors in this group.

Activity other than B_{12} activity is supplied to the thyroid fed rat by yeast and tomatoes (Betheil and Lardy, 1949). Ershoff reports that the effect of whole liver in allowing increased survival of rats on a purified diet with thyroid-active material is shared by yeast, by cholesterol, by a benzene or ethylene dichloride extract of liver, and by xanthine; none of these supplements affect growth appreciably. The growth promoting effect of the whole liver is found in kidney, heart, placenta, duodenum, thymus, and brain; also in the hot water insoluble liver residue. Fish solubles and Bird's cow manure concentrate were not effective (Ershoff, 1948; Ershoff and Marx, 1948).

f. Pork. Evidence that pork is low in some factor needed for reproduction and lactation is provided by the work of Everson *et al.* (1948) and Henderson *et al.* (1948). Reference might also be made to the paper of Cabell and Ellis (1947) which indicates that both alcohol extracted casein and pork are deficient for rat growth, but the deficiency of the pork is *not* remedied by a liver extract which considerably improves the results with casein.

g. Growth Factors for Microorganisms. Two of the many postulated growth factors for microorganisms are of some interest for the present discussion. Protogen, or factor II A (Stokstad *et al.*, 1949b; Kidder and Dewey, 1945), is a liver factor needed by the protozoan *Tetrahymena geleii*, which is of special interest because of the similarity between the nutritional requirements of this organism and of higher animals. It has now been established that protogen concentrates show no B_{12} activity for *L. lactis* Dorner or APF activity for chicks (Stokstad *et al.*, 1949b). Its probable identity with two other factors important for *S. faecalis* and *L. casei* has been pointed out (Snell and Broquist, 1949), but there is as yet no evidence for any role in higher animals.

A factor needed by *Leuconostoc citrovorum* and known by this name also occurs in liver and in antipernicious anemia concentrates of liver (Sauberlich and Baumann, 1948; Sauberlich, 1949). Apparently thymidine can partially substitute for the factor in the nutrition of this organism just as it can substitute for B_{12} in the nutrition of *Lactobacillus lactis* Dorner and *Lactobacillus leichmannii*. Crude concentrates of the citrovorum factor are not effective in rats on purified casein rations containing desiccated thyroid (Betheil *et al.*, 1949) indicating that this factor does not provide either B_{12} activity or the unknown activity of liver residue. Interesting light on other behavior of the citrovorum factor is shed by Lyman's electrophoresis studies (Lyman and Prescott, 1949a, b) and by the paper strip chromatography of Winsten and Eigen (1949b).

VIII. ROLE OF B_{12} IN METABOLISM

1. Protein Metabolism

There is general agreement (see Sections III, 3, c and IV, 2, b) that at high levels of protein in the diet signs of B_{12} deficiency are more marked. This is interpreted as a B_{12} requirement paralleling the protein intake and it is concluded that B_{12} is involved in protein metabolism. However, involvement in protein metabolism may mean many things. There are certain indications that nitrogen disposal is unfavorably affected by B_{12} deficiency. McGinnis *et al.* (1948) showed that an active concentrate from liver lowered the high NPN in chicks on a ration containing no animal protein source. Zucker and Zucker (1948a) found very high NPN values in rats on all-plant rations at the time of the most marked signs of deficiency. The hypertrophied kidneys of normal structure reported from several laboratories may be significant (Zucker and Zucker, 1948a; Cary and Hartman, 1947; Hartman *et al.*, 1949b).

Of greater interest than these general indications are data concerning particular biochemical changes which are affected by the presence or absence of B_{12}. Since in many of the chick diets methionine was a limiting factor, the question whether it could be replaced by B_{12} came up early. This was answered in a negative sense by McGinnis *et al.* (1947b) and by Mishler *et al.* (1948). In these reports the B_{12} source was, respectively, a high potency liver concentrate and fish solubles which certainly carry B_{12} activity whatever else may be present.

Observations of Bird *et al.* (1947) dealing with supplements of the cow manure factor and methionine, indicated that the two requirements were not entirely independent. In a review Bird (1947a) has also dealt in general with the dependence of requirements on the rest of the diet composition. How this applies to the B_{12}-labile methyl relation is illustrated by a number of studies.

Gillis and Norris (1949) fed a basal diet containing the equivalent of about 0.1% choline with no source of B_{12} added. On the addition of either choline or B_{12} in the form of "liver paste," growth of the chicks increased. However, the combination of both produced no better a result than "liver paste" alone because the formerly inadequate level of choline had now become adequate. Schaefer *et al.* (1949b) using a methionine and choline low (peanut and casein) diet studied growth of chicks when several levels of choline and of B_{12} were given. Among the experiments is a good approximate replicate of the above observations of Gillis and Norris. They also show a lower limit for methyl group requirement even in the presence of ample B_{12}, and, in the presence of large amounts of choline, B_{12} is still required. Jukes and Stokstad (1949) present evidence on both a choline deficient (glucose-casein-gelatin) diet and one deficient in methionine (ground peas, gelatin. glucose). Neither choline nor methionine can "replace" the B_{12}. With 6 g. of choline per kg. of diet, or respectively, 4 g. of methionine a B_{12} requirement is still maintained.

Schaefer *et al.* (1949a) in experiments with weanling rats from stock diet mothers studied the results of various concentrations in the diet of choline or methionine and of B_{12} on mortality, renal damage and growth. When their basal labile methyl deficient diet was supplemented with choline, the weanling rats showed no demonstrable need for B_{12}. This is in line with the general experience with rats, that B_{12} deficiency in the mothers' diet (even with quite adequate methionine content) will bring out deficiency signs in the young, while weanling rats simply put on B_{12} deficient diets show barely measurable weight deficits or other signs (see Section III, 3, a). Under stress of the methionine deficient diet, however, a B_{12} supplement improves growth and other conditions.

Regarding mechanisms involved in this phase of B_{12} activity two very recent reports are of particular interest. Bennett (1949) finds that under certain conditions B_{12} can play a decisive role in a methylation process. In a continuation of her studies in rats (Bennett *et al.*, 1944) on the production of methionine from homocystine without dietary sources of labile methyl groups, it is found that in B_{12} deficiency the process does not take place but when pure B_{12} is given methionine is formed, as indicated by resumption of reasonable growth rates. Four micrograms of B_{12} per day gave a larger effect than 2 μg. Stekol and Weiss (1949) essentially concur in Bennett's findings and extend the investigation of B_{12} effects to other phases of the metabolism of methyl groups and sulfur amino acids. These results suggest the possibility that the hitherto unnoticed variable content of B_{12} in stock or experimental diets, the variable storage and perhaps intestinal synthesis may be the key to some puzzling situations which have turned up in methylation studies.

2. Relation to Nucleic Acids

It has been known for some time that in the metabolism of lactic acid bacteria folic acid appears to be concerned with the synthesis of thymine. This is argued from the fact that if thymine is added to the medium in suitable amounts folic acid is no longer required for growth (Stokes, 1944). In the growth of two organisms which require B_{12} (*L. lactis* Dorner and *L. leichmannii*) it was found that large amounts of thymidine can take the place of B_{12} (Shive *et al.*, 1948b; Wright *et al.*, 1948; Skeggs *et al.*, 1948). The interesting question now arose as to what part of the desoxyriboside molecule was responsible for the activity. Further studies (Kitay *et al.*, 1949) revealed that the relation to B_{12} is not specific for thymidine, the other common desoxyribosides acting similarly. In some of the lactic acid bacteria which require B_{12} desoxyribonucleic acid could take its place, but desoxyribose was not active in any so far examined.

According to Cheldelin *et al.* (1949) *Lactobacillus gayoni* requires relatively large amounts of B_{12} as well as thymidine. The flagellate *Euglena gracilis* requires B_{12} for growth but thymidine will not take its place (Hutner *et al.*, 1949). Hutner points out that one is not justified in making generalizations regarding other species on the basis of the model afforded by the lactobacilli.

It has been tacitly assumed that nucleic acids are synthesized in animal tissues from non-characteristic precursors. Neither they nor their components (except phosphoric acid) are in any sense dietary essentials, and suppression of intestinal bacteria has not so far turned up any deficiencies of nucleic acid components.

As far as the folic acid-thymine relation is concerned, Petering and Delor (1949) showed that it does not hold for mammals (rats) at least in so far as the hematopoietic function of folic acid is concerned. In chicks Totter *et al.* (1949) have found that graded amounts of PGA in the diet do not materially affect the desoxyribonucleic acid (or ribonucleic acid) in the liver. The neat speculation of Wright *et al.* (1948) regarding the relation of B_{12} to the nucleic acids of mammals or especially to the pernicious anemia problem will require more data if it is to be rationally revived. The recent results of Reisner and West (1949) neither give any substantial support nor do they rule it out completely.

If the results of Stern *et al.* (1949) on the demonstration in sections of greater cytoplasmic levels of nucleic acids due to B_{12} administration are referable specifically to the supplement and not simply to better growth, a novel method for studying B_{12} activity may be available.

3. *Interrelationship of B_{12} and Folic Acid*

The pernicious anemia syndrome is relieved by B_{12}, but PGA will lead to a remission of the hematological symptoms. B_{12} deficiency in experimental animals in the presence of an adequate supply of folic acid has so far led to no abnormality in the blood picture (Emerson, 1949; Emerson *et al.*, 1949b; Betheil *et al.*, 1949; Neumann *et al.*, 1948; Rubin and Bird, 1946a; Nichol *et al.*, 1949a; Sporn *et al.*, 1947; Williams *et al.*, 1949). In chicks on purified casein diets containing thiamine, riboflavin, pyridoxine, niacin, pantothenic acid, biotin, choline, and vitamins A, D, E and K, and in pigs or rats on such a diet also containing a suitable sulfa drug or PGA antagonist, there is besides a severe growth inhibition a reduction in the circulating cellular elements of the blood, with maturation arrest and hyperplasia of immature elements in the bone marrow, findings which are typically present in human pernicious anemia (Weir *et al.*, 1948). Such diets have long been recognized as deficient in folic acid. We now know that such diets are also potentially deficient in B_{12}. How deficient in B_{12} the animals in any given experiment were depends on the maternal diet, intestinal synthesis, access to feces, degree of purity of casein in the diet, the rest of the diet composition with special reference possibly to riboflavin, etc. However, in all cases, whether B_{12} was deficient or not, the principal deficiency has been that of folic acid, judging by the ability of the pure substance to restore growth and blood picture to normal or near normal.

In recent work (Williams *et al.*, 1949) where B_{12} was undoubtedly deficient as well as PGA, through the use of chicks from hens on a B_{12} deficient plant ration, the growth responded separately either to B_{12} or PGA, the latter having the larger single effect. Thus B_{12} alone increased the gain by 56 g., PGA alone by 175 g., PGA + B_{12} by 219 g. There is no indication that growth effects under these conditions are anything but simply additive, as is often the case with double deficiencies. Similarly the growth of Rous tumor implants in chicks on a purified diet deficient in B_{12} and PGA was stimulated by B_{12} or PGA separately, with a larger effect from the two factors given simultaneously (Oleson and Little, 1949).

However under these conditions of a double deficiency of PGA and B_{12} it has been possible in some instances to demonstrate an effect of B_{12} on hematopoiesis. Thus Welch *et al.* (1948) and Cartwright and Wintrobe (1949) in pigs, Nichol *et al.* (1949b) in chicks, Ruegamer *et al.* (1948a) in dogs, and Emerson and Mushett (1948) in rats have produced anemias or leucopenias which responded promptly to PGA treatment but also showed more or less response to B_{12}. Where a good hematologic response has been produced with B_{12} alone (Welch *et al.*, 1948; Ruegamer *et al.*, 1948a) there was a subsequent relapse which yielded only to PGA. PGA therefore remains the key factor in the animal work for maintaining a normal blood picture, but there is some evidence that B_{12} can also play a role.

The nature of the deficiencies induced in the rat by feeding a sulfa drug needs further attention. According to Johnson and Dana (1949) administration of ascorbic acid cures the leucopenia and leads to a partial weight response, while not affecting the anemia.

A large excess of PGA completely suppresses the action of B_{12} microbiologically (Greeve *et al.*, 1949).

Scott *et al.* (1949) have reported that B_{12} is necessary for the functioning of the enzyme system of chicken liver which liberates PGA from the heptaglutamate.

4. Relation to Carbohydrates and Fat

The effect of B_{12} activity in the presence of excess protein or in deficiency of other essentials such as methionine has been discussed above. Bosshardt *et al.* (1949b) have recently added a new phase to the investigations. In weanling mice from stock diet mothers a quite measurable deficiency of B_{12} can be demonstrated when the fat in the 20% casein diet is largely replaced by carbohydrate. Thus, they indicate, a good response to B_{12} can be elicited without depletion and without stress of a second deficiency. They cite the analogy to other altered B factor effects in response to changes in proportions of the major foodstuffs in the diet. Full publication and further developments along such lines will be awaited with interest. The fact that lower levels of fat are used in poultry diets than is common in most of the rat work may have a bearing on the relative ease of obtaining signs of deficiency. It might even be surmised that high fat levels in diets for mammalian reproduction have a connotation of this kind.

Quite separate from this are the findings of Hartman *et. al.*, 1949b and Cary and Hartman (1947) that growth of rats on their diet containing purified casein is depressed by substituting 25% lactose for dextrin. The detrimental lactose effect is also overcome by the administration of liver extract or B_{12}.

5. Intestinal Synthesis

For both rats and poultry this subject has been discussed above (Sections III, 3, b; IV, 2, d).

It may be that poultry in the wild state would obtain sufficient B_{12}

from soil and various lower animal forms and that the hen faces a B_{12} problem only under the restrictions of domestication. Wholly carnivorous birds of prey will have an automatic supply just as other carnivorous species. With regard to the typically grain or seed eating birds like the pigeon the crop contents ("pigeon milk") might be investigated unless these species have more efficient supplies from intestinal synthesis than do chickens.

When it comes to herbivorous mammals the ruminants certainly have an efficient arrangement. The microbiological processes in the rumen with the high production of B_{12} activity insure full opportunities for good absorption in the rest of the gastrointestinal tract.

In non-ruminant herbivora one would presume that intestinal synthesis and adsorption must be efficient enough. In the horse the cecum is said to have the properties of a fermentation pouch not dissimilar to the rumen; a rather efficient absorption in cecum and colon would have to be postulated. The rabbit and probably the guinea pig have an additional very practical provision. Madsen (1939) and Eden (1940) have pointed out that the rabbit produces two kinds of feces, the well known kind one always sees, called the day feces, and also the night feces which are regularly eaten in the morning. In a further report Eden (1945) draws a parallel between the rabbit's cecum and the rumen of ruminants. The food from the small intestine is divided between the colon (for the day feces formation) and the cecum where an approximately 24 hour fermentation prepares the night feces for reingestion so that the products are subject to full absorption in the small intestine.

When Kunkel *et al.* (1948) fed rabbits on a casein-sugar diet they established a requirement for a liver factor. If such studies in rabbits are continued it may be worthwhile to observe whether there is simply a dietary deficiency or whether the unnatural diet changed the production or possibly even reingestion of bacterially produced factors.

As Williams (e.g., Williams 1943) has repeatedly pointed out, the B factors are rather universally distributed and are all freely available in plant feeds. The particular B factor that we have been taught to call B_{12} is available in less than practical amounts in a large class of foodstuffs. Therefore unless it can be synthesized by the tissues in some species as in the case of ascorbic acid the synthesis by commensals is of greater importance than it is with other nutritional essentials. This we hope warrants the above digression into regions which may be vague and far afield.

6. *Storage in Tissues*

B_{12} differs from the other B factors in distribution in nature, as was discussed above (Section VII, 1, b). There is also a difference in storage behavior. Cravens and Halpin (1949) have pointed out how much less readily chickens are depleted of B_{12} than of other B factors. In rats maternal storage is largely responsible for the late recognition of the existence of the factor. It required observations on offspring of depleted mothers instead of the traditional practice of giving the deficient diet to stock weanlings.

The degree of storage in various species and in various tissues will raise questions of biochemical as well as practical interest. One such problem has already come up with the finding of a much lower B_{12} content in pork than in beef muscle (Register *et al.*, 1949a). The explanation which is offered, namely that the pig is without a rumen, is probably not the whole story since pig liver appears to be about as rich in B_{12} as beef liver. If muscle cannot get much B_{12} without a rumen, how does the liver get it? Do livers have a particular garnering ability and only when there is a surplus production will the muscle also show an appreciable amount? Is it a matter of something like a "partition coefficient," saturation and overflow, or what? Williams (1943) has discussed the distribution of B factors in tissues. For instance, thiamine is high in pork muscle compared with beef muscle, while in all the other tissues of the pig it is low compared to the corresponding tissues of beef. The possible bearing of low B_{12} in pork muscle to the rat feeding experiments of Everson *et al.* (1948) has been mentioned before (VII, 2).

7. *Relation to Cobalt*

When it became known (Smith, 1948b; Rickes *et al.*, 1948b) that B_{12} contains cobalt, there was much speculation concerning the relation of the anemia of cobalt deficiency to pernicious anemia. The literature on cobalt deficiency in ruminants (see e.g., Marston, 1939) indicates that there is no reason to believe that besides the occurrence of anemia the pictures have anything in common. These questions are largely settled by recent data of Becker *et al.* (1949) which indicates that B_{12} is not an important intermediate in the cobalt deficiency syndrome of ruminants. A number of clinical observers have found cobalt *per se* is without any influence on pernicious anemia (e.g. West, quoted in Rickes *et al.*, 1948b). Also (Emerson, 1949) B_{12} deficient rats do not respond to cobalt. It thus appears that cobalt may have two entirely separate functions in animal biochemistry.

There are some observations on cobalt which, on more general grounds, are of interest. Comar *et al.* (1946) using radioactive cobalt, show that when fed to cows it hardly appears in the tissues and is mostly eliminated in the feces whereas injected cobalt is hardly found at all in the gastrointestinal tract and is largely excreted in the urine. Data for distribution in tissues are also given. Gall *et al.* (1949) show that in cobalt deficient sheep some modification of bacterial flora of the rumen can be demonstrated, i.e., that cobalt deficiency may inhibit B_{12} production by inhibiting bacterial growth.

Tosic and Mitchell (1948) determined the cobalt in various fractions of sheep's rumen content. They found in round figures: in the protozoal fraction 1.8 μg./g. of dry substance when hay of good cobalt content was fed and 0.6 with cobalt deficient hay; similarly the bacterial fractions contained respectively 3.8 and 1.5 μg./g. The two kinds of hay contained 0.27 μg./g. and 0.07 μg./g. The values for the B_{12} equivalent in the protozoal (6.3 μg./g. B_{12} = .28 μg./g. cobalt) and bacterial (2.1 μg./g. of B_{12} = 0.09 μg./g. cobalt) fractions of sheep's rumen content obtained by Zucker and Zucker (1948e; see Table II) are probably valid enough for a rough comparison. The indications are that the rumen microorganisms contain a considerable excess of cobalt over that which is contained in the B_{12}, that good hay furnishes a large excess of cobalt over that needed for B_{12} synthesis, and that even the deficient hay at 0.07 μg./g. may furnish enough cobalt for normal B_{12} synthesis in the bacteria. The bacteria which synthesize the B_{12} apparently have, as emphasized by Tosic and Mitchell, considerable ability to concentrate the cobalt from the medium as compared with the protozoa which store B_{12}.

IX. GENERAL SUMMARY

Vitamin B_{12} is the name given to a chemically and physically characterized cobalt coordination compound which has been isolated from liver and from the culture medium of certain molds. It is the antipernicious anemia substance of liver and also plays the role of a nutritional essential for certain microorganisms, for birds and for mammals. Its structure has as yet been worked out only in part. So far three different forms of such a Co coordination compound (B_{12}, B_{12a}, B_{12b}) have been found which have B_{12} activity.

Before its isolation it had been studied as a nutritional factor in chickens, rats and mice under designations such as "animal protein factor," "chick growth factor," "hatchability factor," "nutritional factor X," and "zoopherin." Natural sources in which it had been studied included liver, meat and fish products, dairy products, and also cow manure. It is produced by many bacterial species. It is found in soil, due to organisms as yet unidentified. In chicken droppings it accumulates up to considerable concentrations on standing or incubation.

In both poultry and rats the deficiency manifests itself most clearly in interference with viability of the offspring. In birds the critical time is hatching; in rats and mice it may vary with the severity of the deficiency. Fertilization and egg production in birds and impregnation, parturition, and lactation in rats are not directly affected. The growth of surviving chicks, rats, or mice can be used in measuring B_{12} activity. Offspring of parents on diets containing adequate B_{12} may show little or only delayed effects of a deficiency. The efficient transfer of B_{12} to the young is a factor which lent much uncertainty to the earlier work.

Experimental diets have been principally of two types: all plant rations, i.e., diets made up of components naturally free of B_{12}, and purified diets using casein from which B_{12} has been removed by alcohol extraction. With the former the main consideration is that all other nutritional essentials are provided for, while in the casein diets the thorough removal of the B_{12} adhering to the casein is an additional consideration.

An alternative technique consists of feeding either chicks, rats, or mice on any of a variety of diets with the addition of thyroid substance (or its equivalent). According to Drill, such a procedure increases nutritional requirements and whatever essential is the limiting factor can then be measured with greater ease. Consistent and apparently true results have been obtained for B_{12} assays. There are, however, certain contradictory statements in the literature concerning the applicability of this test for B_{12}. These need to be resolved.

There are many present indications that crystalline B_{12} possesses all the activity which is connoted by animal protein factor as represented by liver, liver extract, fish meal, fish solubles, etc. This is based largely on curative experiments. Critically controlled preventive experiments have not yet been carried out. There are also indications from other lines of experimentation that whole liver and a *Streptomyces aureofaciens* preparation contain materials which are not B_{12} but have activity which may come under a heading of "animal protein factor" activity.

Striking features which have a bearing on problems concerning B_{12} are: its apparently exclusively microbiological origin and, for all practical purposes, its absence from food materials of plant origin; its unusually effective storage; and, in poultry, the easy availability if any access to droppings is permitted. These points have all played a large role in the development of the subject.

With regard to the relation of B_{12} to metabolic processes, it is fairly well established that the requirement or effectiveness of B_{12} will vary with the protein intake and similar relations to fat are indicated. The suggested relation to methylation processes is of the highest interest. Whether in the animal body it plays a role in the synthesis of some of the nucleic acid moieties, such as is indicated in bacterial metabolism, is still an open question.

It is needless to say that the developments are only in their beginnings and are moving fast. All types of assay have presented unusual problems but, on the other hand, there has probably never been such a simultaneously concerted effort utilizing physical methods, microbiological techniques, and practically all animal species that are suitable. When brought to fruition these accomplishments will have large effects on future assay problems. Generally applicable assay methods, when applied to separate tissues of various species, also bid fair to uncover unlooked for relations. Such data should form the basis for pertinent physiological considerations. There are also indications that such further studies in the distribution of B_{12} will lead to new biochemical considerations in animal ecology, to say nothing of the benefits to animal husbandry. To the chemist the apex of the structure will be an understanding of the biochemical mechanisms in which B_{12} plays a role.

We are well aware of the temerity of the attempt to give at this time a satisfactory account of the developments in this field. Although the views and interpretations here presented may become outmoded or possibly trivial when this has barely appeared in print, we believe that this review has a place in recording the steps from the early work to the present. The fact that nearly 15 years intervened between the time the problem was opened up and the constructive recognition of its importance testifies not only to its complexities but also to the insight of the pioneers: the Beltsville group and others in this country and Mapson in England.

Addendum

The text of the review was completed in early October 1949. With no pretense of completeness, the following items tend to round out the picture as of June 1950.

In January 1950 the first extensive paper to appear on B_{12} with details of preparative procedures was published from Glaxo Laboratories ("Crystalline Anti-Pernicious Anaemia Factor from Liver"; Fantes, K. H., Page, J. E., Parker, L. F., and Smith, E. L. 1950. *Proc. Roy. Soc. London* **B136,** 592). Methods used were largely physical. During concentration of B_{12} activity, adsorption and elution behavior, solubility in alcohol, and some liquid-liquid distribution properties change considerably. On the other hand insolubility in near saturated aqueous ammonium sulfate and precipitation with phosphotungstic acid are effective at all stages of purification. Colorimetric estimation of B_{12} is unsafe because of the existence of other red substances which are inactive. There is no significant exchange of the P with radioactive P of phosphate in neutral, acid or alkaline solution, nor with radioactive Co either as cobaltous chloride or as hexammino-cobaltic chloride, indicating that both the Co and the P are firmly bound in coordinate linkage. Physical properties listed for the pure substance include ultra-violet and infrared absorption spectra, optical rotation, electrometric acid-base titration, electrical conductivity, electrical mobility, polarogram, and diffusion properties. In an accompanying paper from Oxford, X-ray crystallographic data are given (Hodgkin, D., Porter, M. W., and Spiller, R. C., *ibid.* p. 609).

The ninhydrin reacting acid hydrolysis product of B_{12} has been identified as D-1-amino-2-propanol (Wolf, D. E., Jones, W. H., Valiant, J. and Folkers, K. 1950. *J. Am. Chem. Soc.* **72,** 2820).

Cobalt can be a limiting factor in the biosynthesis of B_{12} (Hendlin, D. and Ruger, M. L. 1950. *Science* **111,** 541) and a radioactive B_{12} has been obtained by biosynthesis in the presence of the radioactive cobalt^{60} (Chiaet, L., Rosenblum, C. and Woodbury, D. T. 1950. *Science* **111,** 601).

The additional APF factor (see Section III, 4, a above) found effective for chickens in preparations from *Streptomyces aureofaciens* has been identified as the antibiotic aureomycin (Stokstad, E. L. R., and Jukes, T. H. 1950. Am. Chem. Soc., 117th Meeting, p. 12A; date not included in printed abstract). The activity is apparently due to its ability to produce more favorable intestinal microorganisms. It had previously been found that phenyl arsonic acid derivatives which are used for treatment of coccidiosis also stimulate growth in presumably healthy chickens (Bird, H. R., Groschke, A. C. and Rubin, M. 1949. *J. Nutrition,* **37,** 215). Moore *et al.* (Moore, P. R., Evenson, A., Luckey, T. D., McCoy, E., Elvehjem, C. A., and Hart, E. B. 1946. *J. Biol. Chem.* **165,** 437) before the association of B_{12} with some of the antibiotics was known, reported on growth effects when antibiotics and intestinal antiseptics were fed. Streptomycin and sulfasuxidine increased growth while streptothricin was negative. They offer possible interpretations of such results: 1) suppression of organisms which compete successfully with the host for intestinally produced growth factors, 2) suppression of organisms which do not synthesize growth factors in favor of those which do so, 3) inhibition of organisms pathogenic to the host and, 4) possibly also a favorable physiological effect on the host directly. Aureomycin has also shown growth stimulating properties in swine (Jukes, T. H., Stokstad, E. L. R., Taylor, R. R., Cunha, T. J., Edwards, H. M. and Meadows, G. B. 1950. *Arch. Biochem.* **26,** 324) and is apparently responsible for the results previously reported by Cunha *et al.* 1949 (Section III, 4, a). Streptomycin has a similar effect in swine (Luecke, R. W., McMillen, W. N. and Thorp, F. J. 1950. *Arch. Biochem.* **26,** 326). Rats on B_{12} deficient diets do not respond to aureomycin (Zucker, T. F. and Zucker, L. M. 1950. Am. Chem. Soc., 117th Meeting, p. 16A) nor to phenyl arsonic acid (unpub.). The aureomycin effect in chickens and pigs presents possible complications with B_{12} assay in these species. Since the aureomycin acts locally in the intestine and does not enter into metabolic processes of the animal it can hardly be regarded as a nutritional factor.

In well controlled experiments a deficiency, presumably B_{12}, has been produced by feeding 5% sulfasuxidine to rats (Jones, J. H., Rogers, C. S., and Stone, C. H. 1949. *J. Nutrition* **39,** 579). Intestinal synthesis of B_{12} activity in feces (see Section IV, 2, d above) of rats on purified diets deficient in B_{12} has been verified (Zucker, T. F. and Zucker, L. M. 1950. Am. Chem. Soc., 117th Meeting, p. 16A) in the same laboratory, with the same rat stock, where no activity was found in feces of rats on a 60% cottonseed all-plant diet deficient in B_{12}.

It has been reported that alfalfa contains enough saponins to be toxic to chickens (Peterson, D. 1950. *J. Biol. Chem.* **183,** 647). This information should be borne in mind when this species is used in the evaluation of the possible nutritional role of alfalfa. Peeler *et al.*, using microbiological techniques and chemical procedures confirm previous animal experiments (see Section VII, 1, b) to the effect that there is little if any B_{12} in alfalfa. An unidentified factor simulates B_{12} activity (Peeler, H. R., Miller, R. F., Carlson, C. W. and Norris, L. C. 1950. Am. Chem. Soc., 117th Meeting, p. 16A (cf. Peeler *et al.* 1949. *Proc. Soc. Exptl. Biol. Med.* **72,** 515). For further discussion of feeds of plant source in relation to B_{12} see Jaffé, W. Vitamin Hormon Fermentforschung 1949, **2,** 33.

The citrovorum factor is needed to counter the toxic effects of aminopterin (4-aminopteroylglutamic acid) in microorganisms (Sauberlich, H. E. 1949. *Arch. Biochem.* **24,** 224). The factor is found normally in rat urine (Betheil, J. J., Drysdale, G. R., Baumann, C. A. and Lardy, H. A. 1949. Am. Chem. Soc., 116th Meeting, Abstracts p. 35 A) and in human urine (Anker, R. M., Boehme, J. W. and Welch, A. D. 1950. *Federation Proc.* **9,** 351). Thus a metabolic role for this factor has been established in higher animals. Interrelationships of B_{12}, citrovorum factor and desoxyribosides for microorganisms are indicated by the work of Jukes, T. H., Broquist, H. P., and Stokstad, E. L. R. 1950. *Arch. Biochem.* **26,** 157. In the early chick embryo desoxyribosides protect from aminopterin toxicity, while the citrovorum factor is ineffective (Snell, E. E. and Cravens, W. W. 1950. *Proc. Soc. Exptl. Biol. Med.* **74,** 187).

Ungley has found thymidine inactive in human pernicious anemia (Ungley, C. C. 1949. *Lancet* **256,** 164). It has been reported (Wetzel, N. C., Fargo, W. G., Smith, I. H., and Helikson, S. 1949. *Science*

110, 651) that B_{12} stimulates the growth of some under-nourished children; confirmation will be awaited with interest.

Since the original papers on B_{12} in swine nutrition, three more complete reports have now appeared. G. C. Anderson and A. G. Hogan (1950. *J. Nutrition* **40,** 243) and A. L. Neumann, B. C. Johnson and J. B. Thiersch (1950. *J. Nutrition* **40,** 403) produced a deficiency which was relieved by B_{12} while J. E. Burnside, T. J. Cunha, H. M. Edwards, G. B. Meadows, G. A. LaMar, A. M. Pearson and R. S. Glasscock (1950. *Proc. Soc. Exptl. Biol. Med.* **74,** 173) found on their diet no deficiency which was corrected by B_{12}. The APF concentrate from *S. aureofaciens* improved growth, however, and prevented a diarrhea which occurred periodically in the untreated pigs. It may be well to recall here the discussion by Moore *et al.* mentioned above and what has been said in the body of the review on maternal diet and intestinal synthesis.

Additional data on B_{12} activity in natural materials have appeared (Lewis, U. J., Register, U. D., Thompson, H. T. and Elvehjem, C. A. 1949. *Proc. Soc. Exptl. Biol. Med.* **72,** 479).

Marked growth stimulation in rats with as little as 0.05 μg B_{12} per day (administered 3 times per week) has been reported by Frost, D. V., Fricke, H. H., and Spruth, H. C. 1949. *Proc. Soc. Exptl. Biol. Med.* **72,** 102. Some of the work was done using the same diet on which a requirement of 0.5 μg per day (given daily) was found in our laboratory (see Table IV). Reasons for such discrepancies need to be investigated.

Some B_{12} data were reviewed by Wintrobe (lecture before the Harvey Society, N. Y., Jan. 19, 1950). There was another symposium on B_{12} at the 117th Meeting of the American Chemical Society, April 1950, of which abstracts are available.

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Pyridoxine and Fat Metabolism*

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I. INTRODUCTION

Goldberger and Lillie (1926) reported the production of acrodynia in rats fed a diet deficient in vitamin B_2. Later, Ohdake (1931) isolated a compound with the formula $C_8H_{11}O_3N \cdot HCL$ from rice polishings; however, he did not recognize this compound as a vitamin. To György (1934) goes the credit for establishing vitamin B_6 as a new entity in the vitamin B complex. In 1938 five different groups of investigators (Lepkovsky, 1938; Keresztesy and Stevens, 1938; György, 1938; Kuhn and Wendt, 1938; Itiba and Miti, 1938) independently isolated crystalline vitamin B_6 (pyridoxine) from natural sources. The chemical structure of pyridoxine was determined and it was synthesized during the following year (Harris and Folkers, 1939).

Snell *et al.* (1942) reported that one or more substances occur naturally which have a greater activity than pyridoxine in promoting the growth of lactic acid bacteria. Later, Snell (1944) identified these substances as pyridoxal and pyridoxamine.

In 1949, the American Institute of Nutrition adopted the recommendation that "the term vitamin B_6 be used as a group name to include pyridoxine, pyridoxal and pyridoxamine, and that these specific names be used only where the corresponding chemical compound is meant." (Am. Inst. Nutrition, 1949.) In conformity with this decision the terms vitamin B_6 and pyridoxine are not used synonymously in this paper.

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During the eight-year period beginning 1934, it was shown that vitamin B_6 has an important role in blood, nerve, and skin metabolism. Vitamin B_6 deficiency in animals induces several types of anemia (Fouts *et al.*, 1938; McKibbin *et al.*, 1942), epileptiform fits (Chick *et al.*, 1940; Wintrobe *et al.*, 1942) and characteristic lesions of the skin (György, 1934; Birch *et al.*, 1935; György and Eckardt, 1939). Although the mechanisms whereby these pathological conditions are produced are not yet fully understood, there has been considerable progress in their elucidation.

The biochemical interrelationships between vitamin B_6 and protein and fat have been studied. Pyridoxine, pyridoxal, and pyridoxamine play an important part in protein metabolism. It has been fairly well established that these compounds are involved in the metabolism of tryptophan (Lepkovsky and Nielsen, 1942; Reid *et al.*, 1944; Umbreit *et al.*, 1946), the decarboxylation of amino acids (Gale, 1946) and the transamination of amino acids (Schlenk and Snell, 1945; Bellamy *et al.*, 1945). No such specific reactions between vitamin B_6 and individual fatty acids during metabolism have yet been demonstrated, although there are indications that interrelationships do exist.

The present review is an evaluation of the literature bearing on the physiological and biochemical interrelationships between vitamin B_6, especially pyridoxine, and fat metabolism.

II. Pyridoxine and Fatty Liver Formation

Halliday (1938) observed that the livers of pyridoxine-deficient rats were significantly heavier than those of normal rats, and that they contained two to three times as much fatty acid. The administration of choline to these deficient animals reduced the amount of liver fat slightly, but even huge doses (220 mg. in 11 days) failed to lower it to a normal value. Peretti (1942) also noted that the livers of rats contained more fat when pyridoxine was omitted from the diet. However, neither Gavin and McHenry (1940) nor Forbes (1941) were able to confirm this observation. Their failure was probably due, at least in part, to the shorter test period (3–4 weeks) they used, for McHenry and Gavin (1941) later showed that pyridoxine had lipotropic activity under special conditions. Also Engel (1941) observed that the absence of pyridoxine from the diet of rats had no effect on liver fat over a 3-week period, but provoked a definite rise after 7 weeks, which was not correlated with food intake. Choline was ineffective in curing these fatty livers even in doses of 10 mg./day (Engel, 1942). The lipotropic activity of choline was also less in fat-deficient rats than in those that received the essential fatty acids (Engel, 1942). Engel summarized his results as follows: "Pyridoxine and the essential fatty acids act mutually in influencing the lipotropic action of choline and are complementary in this respect."

III. Pyridoxine and the Oxidation of Fatty Acids

The *in vitro* oxidation of sodium butyrate by kidney slices was greatly accelerated by the addition of pyridoxine (Peretti, 1945a). This effect appeared to be specific, for the addition of pyridoxine did not influence the oxidation of glucose (Peretti, 1945b). Though this finding may indicate that pyridoxine functions in the oxidation of short-chain fatty acids, more work in this field is necessary before any definite conclusions can be reached.

Champougny and Le Breton (1947a) have presented data which suggest that pyridoxine, as well as niacin and pantothenic acid, function as coenzymes during the oxidation of long-chain fatty acids. Using the Thunberg technique, they showed that the dehydrogenation of fatty acids by purified enzyme systems, that were isolated from beef or rabbit liver or rat adipose tissue, proceeded only in the presence of these vitamins. Moreover, pyridoxine, niacin, and pantothenic acid were inactive unless they had undergone a preliminary phosphorylation (Champougny and Le Breton, 1947b).

IV. The Synthesis of Fat From Protein

The first convincing experiments demonstrating the ability of the higher animals to convert protein into fat were performed by Longenecker (1939) and Hoagland and Snider (1939). In both cases, yeast was supplied in the diet as a source of the B-vitamins. McHenry and Gavin (1941) confirmed the above results using crystalline B-vitamins and, in addition, demonstrated that pyridoxine was essential for this synthesis. Although the interpretation of the results of this experiment was criticized by Mitchell (1942), in the light of more recent work, it is believed to be essentially correct. The demonstration that pyridoxine, as pyridoxal phosphate, functions in the decarboxylation (Gale, 1946) and transamination (Bellamy *et al.*, 1945) of amino acids may indicate that these reactions are essential for the conversion of protein to fat.

V. PYRIDOXINE AND THE UNSATURATED FATTY ACIDS

Although the above data indicate that pyridoxine plays a general role in fat metabolism, a more explicit interrelationship has been shown to exist between this vitamin (and its derivatives) and the unsaturated fatty acids. This has already been seen in the effect of pyridoxine and the essential fatty acids upon the lipotropic activity of choline.

1. Egg White Injury

There is a similarity in the external symptoms resulting from egg white injury (Parsons, 1931), essential fatty acid deficiency (Burr and Burr, 1929) and pyridoxine deficiency diseases (György, 1934). MacKay and Barnes (1941) investigated the influence of pyridoxine and corn oil upon the dermatitis produced by the inclusion of commercial dried egg white in the diet of white rats. They found that the dermatitis developed more slowly with the feeding of corn oil instead of Crisco. It is pertinent to note that corn oil is richer in essential fatty acids than Crisco. Moreover, all the signs of the egg white disease, not cured by the corn oil, disappeared when pyridoxine was injected into the animals receiving the corn oil. It was known at that time that egg white dermatitis could be cured by feeding biotin (Birch and György, 1939). Later it was shown that unsaturated fatty acids could replace biotin in the nutrition of certain microorganisms (Williams and Fieger, 1947) and the yellow fever mosquito (Trager, 1948). The experiment by MacKay and Barnes (1941) can be interpreted now as an indication that pyridoxine and essential fatty acids act mutually to enhance or replace biotin.

2. Oleic Acid

During the investigation of the dietary factors which influence the assay of pyridoxine using rats, Sarma *et al.* (1947) showed that the addition of 5% oleic acid to a diet low in pyridoxine (50 μg. per 100 g. of diet) caused a significant depression in growth. This effect of oleic acid was counteracted by the addition of 250 μg. pyridoxine per 100 g. of diet. Since no other fatty acids were tested, it is not possible to state whether oleic acid is specific in this respect. However, the evidence demonstrates the importance of unsaturated fatty acids when submarginal amounts of pyridoxine are fed.

3. The Essential Fatty Acids

Burr and Burr (1929) produced a deficiency disease in rats in 70–90 days by excluding fat from the diet. It was characterized principally by the development of scaly tails and scaly paws, poor growth, kidney lesions, hematuria, and early death. Small amounts of linoleic, linolenic, arachidonic, decosahexenoic, and hexahydroxystearic acids have been reported to cure the symptoms of this fat deficiency (Burr and Barnes, 1943). Turpeinen (1937; 1938) compared the relative efficiencies of linoleic acid and arachidonic acid in producing a maximal growth response, and found that arachidonic acid was three times as potent as linoleic acid. György (1934), and later, György and Eckardt (1939) described a deficiency disease in rats with similar dermal symptoms, which could be produced at a more rapid rate by eliminating pyridoxine from the diets of rats.

The first indication of a possible relationship between pyridoxine and essential fatty acids in the cure of this dermal syndrome came from the studies of Hogan and Richardson (1934). They reported that all rats receiving irradiated yeast in a low fat diet developed a severe dermatitis and died. When 13% fat (hydrogenated cottonseed oil, milk fat, or lard) was substituted for an equal amount of carbohydrate (sucrose), the results were less consistent. The appearance of the dermatitis was delayed, and some rats died without developing any dermal abnormalities. Later, they showed that this dermatitis could be cured by wheat germ oil (Hogan and Richardson, 1935).

The description of the dermal symptoms was very similar to that of a pyridoxine deficiency. In view of more recent data (Rosenberg, 1942) it is evident that the irradiation of the yeast with ultraviolet light destroyed the pyridoxine in the vitamin supplement. Birch and György (1936) observed that linseed oil or lard exerted a sparing action on pyridoxine. Subcurative amounts of this vitamin, given to rats on low-fat diets, produced cures when small amounts of linseed oil were fed concurrently.

Birch (1938), in a more intensive study of this interrelationship, noted the influence of pyridoxine on the fat-deficiency disease, and the influence of fat in a pyridoxine-deficient diet upon the development of the acrodynialike dermatitis. The inclusion of pyridoxine in fat-free diets alleviated the deficiency symptoms, but did not cure them. The addition of essential fatty acids to pyridoxine-free diets tended to delay the onset of the dermatitis and also to decrease its severity. These observations agreed with those of Bender *et al.* (1936) who fed pyridoxine deficient diets and noted that a reduction of the fat (Crisco) content of the diet from 10% to 3% hastened the onset of the dermatitis and decreased the survival time of the rats. The incidence and severity of the dermatitis were the same when the rats were fed fat-free, pyridoxine-free diets as when they were fed a diet containing 3% Crisco. The inclusion of 10% of this hydrogenated fat in the diets delayed the incidence of the dermatitis; 20% Crisco markedly reduced it (Supplee *et al.*, 1940). Sherman *et al.* (1949) observed that 14% hydrogenated fat spared pyridoxine, for the development of the skin syndrome associated with a pyridoxine deficiency was retarded, and the cure of symptoms was hastened when the fat was fed. The addition of fat to these pyridoxine-deficient diets also resulted in better growth during the development and cure of this condition.

Salmon (1938) has observed this supplementary action of essential fatty acids in curing the dermatitis of pyridoxine deficiency. However, these acids were not curative when pyridoxine was omitted from the diet. Gross (1940) has reported similar results. It is interesting that cod liver oil is ineffective (Salmon, 1938; Quackenbush *et al.*, 1939) and that linseed oil is less effective than corn oil. This latter observation indicates that linolenic acid is less active in this respect than linoleic acid. Therefore, the activity of these essential fatty acids is not proportional to degree of unsaturation.

The mutual sparing action of fats and pyridoxine in the production and cure of acrodynia resulting from a fat or pyridoxine deficiency has been demonstrated repeatedly. Schneider *et al.* (1940) showed that rats on a fat-free pyridoxine-free diet, containing only thiamine and riboflavin of the B-complex, developed an acute acrodynia in 4 to 5 weeks. This condition could be cured by the administration of oils containing polyunsaturated fatty acids or a rice bran concentrate. The effectiveness of the rice bran concentrate was dependent in part upon its pyridoxine content and upon a "filtrate factor" obtained by treatment with fuller's earth. These workers also demonstrated that the Burr syndrome, produced with diets containing small amounts of dried brewers' yeast, was ameliorated by the addition of increasing amounts of this yeast. Quackenbush *et al.* (1939; 1942) administered pyridoxine to rats on fat-free pyridoxine-free diets, and were able to arrest the development of the acute signs of the double deficiency. The survival time was extended and a chronic form of the deficiency was produced. When linoleic acid was fed in subcurative doses (5 mg. daily), the effect was similar to that produced by feeding pyridoxine. When the level of linoleic acid was raised to 10 mg. per day, the deficiency disease was cured. It should be noted that subcurative doses of linoleic acid became curative when supplemented with pyridoxine. Thus, there appears to be a synergistic action between these two compounds, although linoleic acid may be the "primary indispensable factor." They also showed that pyridoxine and pantothenic acid caused a more rapid cure than pyridoxine alone, but still not as satisfactory as linoleic acid or rice bran concentrate. They suggested that the "filtrate factor," which Schneider described, consisted of pantothenic acid and at least one other water-soluble compound. This other accessory factor may prove to be biotin. The above data also indicate that at least four compounds (pyridoxine, pantothenic acid, an unknown water-soluble factor, and linoleic acid) are involved in the production and cure of this complex nutritional dermatosis. Richardson *et al.* (1941) have implicated pantothenic acid in the cure of this dermatitis and state that both the water-soluble factors and the essential fatty acids are necessary for complete cure.

In an excellent review on the general significance of essential fatty acids, Burr (1942) has noted that their relative ability to cure a simple fatty acid deficiency depends upon the criterion used, i.e., growth effect, skin effect, water consumption. Thus far, only linoleic acid and arachidonic acid have been reported to clear the skin, promote growth and reduce water consumption to normal. Linolenic acid and cod liver oil fatty acids promote growth, but are relatively ineffective otherwise. It is generally held that in an uncomplicated fatty acid deficiency disease arachidonic acid is most potent in terms of growth, but that linoleic acid is most effective in alleviating the dermal symptoms. The efficiency of the essential fatty acids in sparing pyridoxine as measured by retardation of the development of scaly tails and scaly paws is as follows: linoleic (most active), arachidonic (intermediate), linolenic and fish oil fatty acids (least effective).

The bulk of the available literature on pyridoxine and the essential fatty acids indicates that a synergistic relationship exists between them in maintaining the integrity of dermal tissue and in promoting growth. It is reasonable to assume that since one compound cannot replace the other, each has a specific function to perform in the body, and is merely aided by the other. Therefore, each factor must be regarded as "indispensable."

Emerson (1941) designed experiments to test the possible sparing action of linoleic acid upon the pyridoxine requirement of the rat when fed at subcurative levels, but could not cure the resulting dermatitis. Burr (1942) suggested that the poor growth of Emerson's rats may indicate the presence of another deficiency, and that the higher humidity of her laboratories may have interfered with the dermal tests. Daniel *et al.* (1942) have produced a convulsive syndrome in young suckling rats by feeding pyridoxine-deficient diets. This syndrome was not affected by feeding corn oil to the young deficient rats, but the survival period was prolonged. This finding may indicate that the pyridoxine-sparing action of the essential fatty acids is limited primarily to the dermatitis symptoms and to growth.

Kummerow *et al.* (1948) have offered additional proof that the essential fatty acids are the active components of fats that supplement pyridoxine in the cure of rat acrodynia. Oxidized methyl linoleate was less active than the freshly distilled ester and "drastically" oxidized ethyl linoleate was inactive. It appears that the sparing action of linoleic acid is proportional to the degree of integrity of the double bonds in both the 9:10 and 12:13 positions. Double bonds in the 9:10, 11:12 or 10:11, 12:13 positions do not confer sparing activity upon the respective acids.

Although pyridoxine deficiency has been investigated primarily with rats, other species have been used. In these studies little or no attention has been given to the influence of fat in the development of the deficiency syndrome. Shwartzman and Strauss (1949), working with weanling male Syrian hamsters, were able to show that the onset of the deficiency symptoms, although somewhat different from those of the rat, was delayed when corn oil replaced hydrogenated fat in the diet. However, they reported polyunsaturated fatty acids were ineffective in curing or alleviating the pyridoxine deficiency once it had been established.

Thus far, in this review, the interrelationship between pyridoxine and fat in metabolism has been discussed primarily from a physiological and biological point of view. In more recent years a different mode of attack on this problem has been employed. This centers about the qualitative and quantitative biochemical interactions between pyridoxine and fats. Quackenbush and Steenbock (1942) found that rats deficient in pyridoxine and pantothenic acid had less body fat of a higher degree of unsaturation than normal rats. The feeding of these vitamins caused a threefold increase in the total amount of body fat; it also decreased the iodine number. Similar results were obtained by Kummerow *et al.* (1948). Longenecker *et al.* (1940) observed that when fat-free diets containing none of the B-complex factors were fed, the body fat was more unsaturated than that of rats receiving a complete diet. Medes and Keller (1947) produced a higher unsaturation of body fat by excluding only pyridoxine and fat from the diet of rats.

Sherman (1947) reported that the carcass fatty acids of pyridoxine-deficient rats were more unsaturated than those of normal rats. The addition of as much as 14% hydrogenated fat or peanut oil to these diets did not alter this result. The carcasses of these deficient rats contained one-eighth to one-tenth the amount of fat present in the carcasses of animals receiving pyridoxine, regardless of the presence of 14% fat in the diet. However, on a body weight basis, the per cent fat in pyridoxine-deficient animals was one-third that found for normal animals, indicating that the deposition of fat lagged far behind weight increase. Pyridoxine deficiency also interfered with the efficiency of food utilization; this observation was independent of the amount or kind of fat in the diet.

However, the presence of fat in the pyridoxine-deficient diets improved diet utilization.

A more specific biochemical relationship has been shown to exist between pyridoxine and the essential fatty acids, especially arachidonic acid. The published data demonstrate that under certain conditions arachidonic acid can be synthesized by the growing rat (Eckstein, 1929; Spadola and Ellis, 1936). Sinclair (1932; 1936; 1940) has repeatedly stated that the rat can synthesize the essential fatty acids, in fact, "all the fatty acids usually found in animal tissue." Medes and Keller (1947) have also presented data which suggest that the rat can synthesize essential fatty acids. Sherman *et al.* (1947) demonstrated that the adult rat synthesized arachidonic acid from deuteriumated iso-oleic acids of hydrogenation. However, other workers (Bernhard and Schoenheimer, 1940; Bernhard *et al.*, 1942) using the deuterium technique, have shown that linoleic and linolenic acids could not be synthesized. The present status of this very important relationship is still quite confusing and warrants further research, especially since it has been suggested that the more highly unsaturated fatty acids, such as arachidonic and clupanodonic, may arise secondarily from linoleic and linolenic acids (Nunn and Smedley-MacLean, 1938).

Inasmuch as a relationship has been shown to exist between pyridoxine and the essential fatty acids in the production and cure of rat acrodynia, and since arachidonic acid can be synthesized by the rat, the role of pyridoxine in the metabolism of arachidonic acid has been investigated by Sherman (1947). He found that the carcass fatty acids of pyridoxine-deficient animals contained a higher percentage of arachidonic acid than the fatty acids of normal control animals. Previously, Sinclair (1935) found that certain tissues retain the more highly unsaturated fatty acids during starvation and inanition. Also, Longenecker *et al.* (1940) and Quackenbush and Steenbock (1942) found that animals fed fat-free and vitamin B complex-free diets produced a more highly unsaturated fat than control animals. These observations may indicate that under conditions of abnormal metabolism the rat spares the breakdown of its unsaturated fatty acids and metabolizes other fatty acids preferentially. Pyridoxine also appears to play an important role in this sparing action. Moreover, the higher percentage of arachidonic acid in the fat of pyridoxine-deficient animals suggests that, in this deficiency at least, the sparing action applies primarily to this polyunsaturated acid. Further research is necessary before any definite conclusions can be made. The quantitative analyses of Medes and Keller (1947) in a somewhat similar study show definite progress in this direction.

Although the fat of pyridoxine-deficient animals contains a higher percentage of arachidonic acid than the fat of normal animals, the total amount of this acid is much lower (Sherman, 1947). Weanling rats fed this deficient diet doubled their initial weight in 10 weeks, but the amount of arachidonic acid was essentially the same as that present at the beginning of the experiment. The presence of fat in the diet did not alter the above observation even when lard, containing 0.11% arachidonic acid, was one of the fats. When pyridoxine was added to these diets, and fed to other groups the amount of arachidonic acid increased three- or fourfold within the same period of time. Since there was no arachidonic acid in any of these diets except in that containing lard, it is suggested that arachidonic acid was synthesized. Similar results were obtained by Medes and Keller (1947) using fat-free diets. Pyridoxine, therefore, appears to play a role, not only in the synthesis of arachidonic acid, but also in its deposition.

Although the retention of this polyunsaturated fatty acid by the rat is intimately connected with the metabolism of pyridoxine, other vitamins are also undoubtedly involved (Harris *et al.*, 1948). Further work on this intriguing and important problem is urgently needed.

VI. PYRIDOXINE AND FAT METABOLISM IN ARTERIOSCLEROSIS

It has been known for many years that the development of arteriosclerosis is closely linked to the metabolism of fats. "The mechanism of fat deposition is of particular importance because of the deposition which occurs in arteries in atherosclerosis. The presence of fat is a common characteristic of internal sclerotic plaques, especially in early lesions of atherosclerosis" (Simms *et al.*, 1947). Following the infiltration of the arterial wall by lipide, the affected areas become calcified (Best and Taylor, 1940). By incubating adult chicken arteries *in vitro* with substances which could be absorbed by living cells and converted into visible fat, Simms *et al.* (1947) produced fat droplets within the intima of the arterial wall in a manner "identical with that found in spontaneous atherosclerosis." More recently, Becker *et al.* (1949) have studied a possible correlation in the pathogenesis of atherosclerosis between age and fat absorption. They observed that old people, in whom the incidence of atherosclerosis is most common, absorb fat more slowly than young people and show an increased chylomicronemia following the ingestion of a fat-containing meal, which is more intense and more lasting than that observed in young people. This observation will be of great importance if it can be demonstrated that the sustained presence of particulate fat particles in the blood leads to atherosclerosis, a theory which has been advanced by Moreton (1947; 1948).

Rinehart and Greenberg (1949) maintained five immature monkeys

(*Macaca mulatta*) on a synthetic pyridoxine deficient diet for 5½ to 16 months. All animals developed a moderate leukopenia and anemia, and the pyridoxine content of the blood was lowered from an average normal value of 11 μg. per 100 ml. of blood to values which ranged between 1.5–4 μg. per 100 ml. Most animals developed "some fissuring of the epithelium of the palms of the hands and feet." Sclerotic lesions developed in the arteries of all animals; the condition was more severe in those monkeys which had been longer on the deficient diet. The lesions were prominent in the coronary arteries, as well as in those of the kidney and pancreas. Advanced changes were found in the arteries of the testicle of one animal. The authors state that "it was somewhat surprising to see an advanced arteriosclerosis in the vessels of an immature testis." This observation is important because it suggests that pyridoxine plays a role in the development of arteriosclerosis which has hitherto been unsuspected. Furthermore, it is another illustration of an interrelationship between fat and pyridoxine metabolism.

VII. Possible Mechanisms of Interaction

As mentioned earlier, no information is yet available regarding the exact biochemical nature of the interrelationship that exists between pyridoxine and fat metabolism. It appears that this vitamin is actively engaged in many phases of fat metabolism, its transport, deposition, oxidation, and synthesis. Although pyridoxine is involved in the general metabolism of saturated and unsaturated fatty acids, it seems to be more intimately associated with the metabolism of the unsaturated fatty acids and especially the metabolism of "essential fatty acids." The animal utilizes these acids more efficiently in the presence of pyridoxine, for in its absence the animal apparently cannot metabolize these acids properly. Instead, he "spares" them by metabolizing other fatty acids preferentially.

Several theories have been proposed to explain the mechanism of this interrelationship. Hove and Harris (1946), drawing a parallel from the sparing action of tocopherols on the essential fatty acids, suggested that pyridoxine acted in the body as an antioxidant. The chemical structure of pyridoxine indicates that it should have this property. These authors incubated a solution of vitamin A in fish oils with 0.08% pyridoxine, as the free base, and noted that the stability of the vitamin A was doubled. Although this mechanism may conceivably explain the ability of pyridoxine to extend the effectiveness of the essential fatty acids, it does not seem to account for its lipotropic activity, nor does it explain its role in the synthesis of fatty acids.

Birch (1938), after comparing some of the similar chemical and biochemical properties of pyridoxine and choline, suggested that pyridoxine might combine with unsaturated fatty acids, in much the same manner as choline with fatty acids in forming lecithin-like compounds. These pyridoxine-containing complexes could function like lecithin, as fatty acid carriers or as essential components of tissue. Inositol is another member of the vitamin B complex with lipotropic activity; it has been shown to be a constituent of phospholipids (Folch and Woolley, 1942). Birch's suggestion may be tested by the isolation of a pyridoxine-containing phospholipid, a compound which has not as yet been obtained. If pyridoxine is combined with fatty acids to form a phospholipid, this compound must be firmly bound to tissue, for it has not been removed as such by fat solvents. A preliminary tissue autolysis is required for the quantitative extraction of pyridoxine (Atkin *et al.*, 1943).

The recent findings that pyridoxine functions as pyridoxal phosphate as part of an enzyme system in the metabolism of amino acids prompts the suggestion that pyridoxine has a similar function in the metabolism of fatty acids. Pyridoxine may exist in animal tissue as a prosthetic group combined with protein. The proof of such a role would add pyridoxine to the long list of B-vitamins that function in the body as an integral part of enzyme systems.

VIII. Summary

A biochemical interrelationship exists between pyridoxine and fat metabolism. The exact nature of this interrelationship is not yet known. Many phases of fat metabolism—transport, deposition, oxidation, and synthesis—are influenced by pyridoxine. The unsaturated fatty acids, especially linoleic and arachidonic acids, seem to be most intimately involved in this interrelationship. Many pathological conditions are induced by abnormal fat metabolism resulting from pyridoxine deficiency, but only one, disorders of the skin, has been studied intensively.

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Physiological and Chemical Aspects of the Antistiffness Factor Essential for Guinea Pigs

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I. INTRODUCTION

A deficiency syndrome, of which stiffness is an outstanding symptom, has been demonstrated in guinea pigs raised on certain diets. Subsequently numerous publications concerning this deficiency have appeared.

Since the original demonstration, research has followed mainly these three lines. (1) The deficiency syndrome has been mapped out in some detail, and the metabolic changes during the deficiency have been studied. (2) The presence of the nutrilite in a considerable number of food substances has been demonstrated. (3) Chemical investigations on the concentration of the nutrilite have been carried out. Further work has been done on the elucidation of the structure of this factor.

The subject of this review has had some controversial aspects. There have been some flat denials that the condition exists (Homburger and Reed, 1945; Kon *et al.*, 1946) and some published notes that indicated doubt (Anon., 1943, 1947, 1948). It should be stated here that some of these critical conclusions were not on all occasions the result of exhaustive study.

Other independent laboratories (Oleson *et al.*, 1947; Caldwell, 1948; Petering *et al.*, 1948) have confirmed the existence of the syndrome, and the curative power of concentrates of the nutrilite.

II. DIETS

During a series of investigations on the nutrition of the carnivorous planarian worm it was found advisable to develop a colony of guinea pigs of known nutritional history from which tissues could be derived to feed the worms. The diet of the guinea pigs was controlled as to vitamin content and it was soon manifest that the tissues produced in this way varied in their effect on the worms according to the vitamin supplements given the guinea pigs (Bahrs and Wulzen, 1936).

The first diet used (Wulzen and Bahrs, 1936) had the following composition:

TABLE I

Barley-Skim Milk Diet

Ground barley	40 %
Baked skim milk	26 %
Clarified butter	10 %
$CaCO_3$	3 %
NaCl	1 %

It was fed to the guinea pigs for a month before the experiment with the worms began. The diet was fortified with enough orange juice or tomato juice to prevent the onset of scurvy in the experimental animals. However, liver, kidney, and heart tissue of these animals were deficient in a nutrilite essential for planaria, since they failed to develop normally when fed on these tissues. Spleen tissue of guinea pigs maintained on the above regime did not become deficient in the planarian nutrilite during the time of the experiment. Supplementation of the diet with grass or kale restored the growth promoting capacity of all tissues and it was concluded that these greens contained a dietary factor, essential for planaria (the "pl" factor), (Figs. 1, 2).

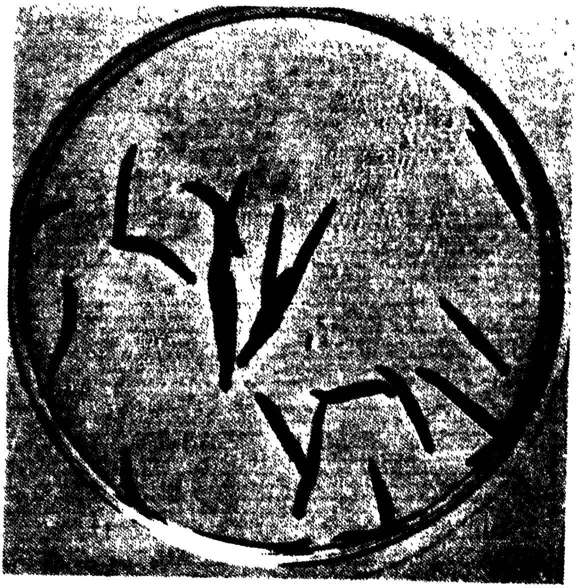


FIG. 1. A group of thirty planaria after 6 weeks' feeding on liver derived from guinea pigs fed the diet specified in Table I, supplemented with fresh kale, 10 g. per animal per day.

Other diets also characterized by the absence of greens, but fortified with ascorbic acid (Bahrs and Wulzen, 1936) also caused a deficiency of the "pl" factor in various tissues (heart, liver, kidney) of guinea pigs and rabbits.

In all these experiments the emphasis had been placed on the "pl" factor. Guinea pigs, furnishing the tissues which produced the deficiency disease in planarian worms had never been kept for longer than about two and a half months on the diet, and had never developed any demonstrable signs of a nutritional deficiency. However, dramatic results were obtained when the animals were kept on the diet for longer periods of time (Wulzen and Bahrs, 1936). Several other diets were tried and a typical one is given below:

TABLE II

Modified Barley-Skim Milk Diet

Baked milk powder	25%
Ground barley	42%
Bran	23%
Yeast	5%
Cod liver oil	3%
NaCl	1%
$CaCO_3$	0.5%
Ferric ammonium citrate	0.002%

The diet was supplemented with 1 ml. of cod liver oil and two drops of viosterol twice per week. The animals received in addition 15 ml. of fresh orange juice or tomato juice daily. The animals were allowed to feed *ad libitum.*

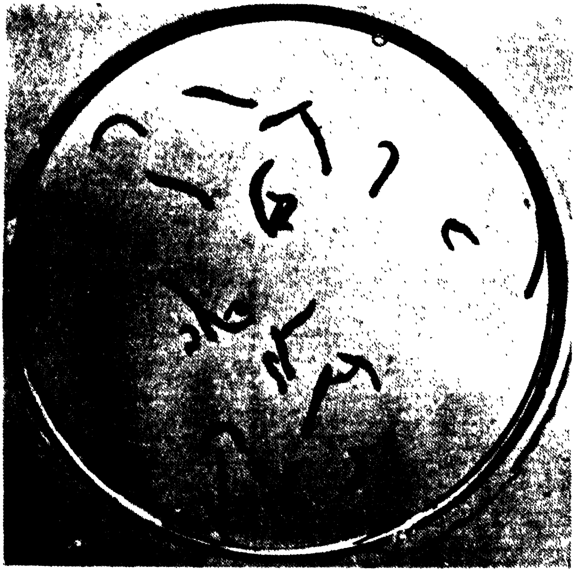


FIG. 2. A group of thirty planaria after 6 weeks' feeding on liver derived from guinea pigs fed the diet specified in Table I, supplemented with fresh tomato juice, 10 cc. per animal per day.

Guinea pigs, receiving no other supplement than orange juice or tomato juice gradually sickened and died after approximately the ninth month. A distinctive syndrome characterized the development and the course of the disease. An early sign was the lowering of muscle tone, so that the animals remained flaccid when handled. The "righting time" was considerably prolonged and at length they could no longer right themselves when placed on their backs. They developed difficulty in moving about the pen, because the hindlimbs could not be manipulated well. In the final stages of the deficiency the animals gradually stiffened until both fore and hind limbs were thrust caudally in extreme extension. The legs were very strongly adducted, and the posterior limbs could not have been spread apart without tearing the adductor muscles. The head was drawn back so that the occiput was pressed against the shoulderblades. It was impossible to press the head downward, and the whole body was so stiff as to be completely immobilized (Fig. 3). None of these symptoms developed when *ad. libitum* supplements of kale or alfalfa were given. If the disease had not progressed too far, administration of these greens restored the animals to a normal condition.

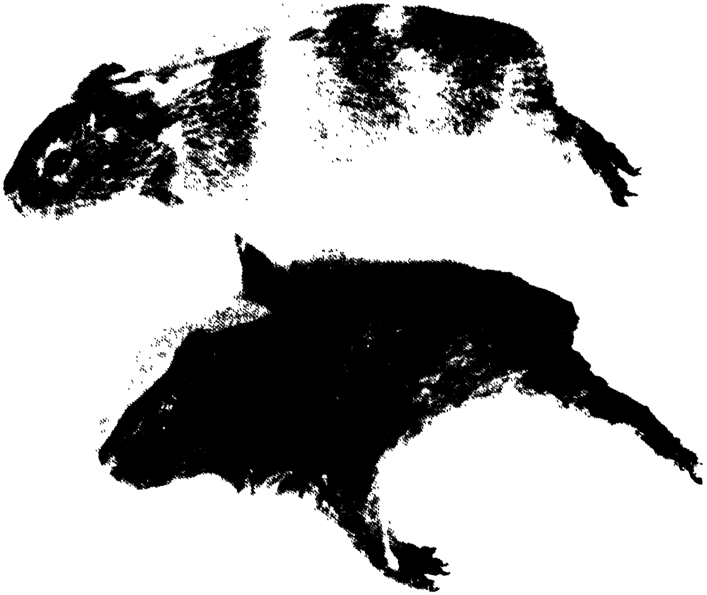


FIG. 3. Live guinea pigs, fed diet specified in Table II for 6 months, until they became unable to move. They were then fed the same diet by hand for 2 months. Their bodies were completely immobilized, the head and legs having the permanent forced positions shown.

Wriststiffness in the early stages of the deficiency could also be induced by a diet consisting of pasteurized whole or skim milk to which 10% of skim milk powder and adequate amounts of carotene and ascorbic acid (in the form of orange juice) had been added (Wulzen and Bahrs, 1941). The final diet used during the course of the later experiments was the following (van Wagtendonk, 1944):

TABLE III

Skim Milk Diet

Skim milk powder	16 g.
Copper sulfate	0.25 mg.
Ferric chloride	0.25 mg.
Water	84 g.

Food was prepared twice a day and evenly distributed over the day and night feeding. To the morning diet was added a solution of the water-soluble vitamins in such a concentration that the average daily vitamin intake was as given in Table IV.

TABLE IV

Water-Soluble Vitamins

Thiamine hydrochloride	0.2 mg.
Riboflavin	0 5 mg.
Pyridoxin hydrochloride	0.1 mg.
Niacin	1.0 mg.
Ca-pantothenate	0.1 mg.
p-Aminobenzoic acid	10 mg.
Choline	50 mg.
Biotin (contained in S.M.A. concentrate, S200, also containing folic acid)	0.01 mg.

A solution of the fat-soluble vitamins in cottonseed oil was either added to the evening food, or fed orally every day. The average daily intake of these vitamins is given in Table V:

TABLE V

Fat-Soluble Vitamins

γ-Carotene	150 I.U.
Viosterol	40 I.U.
α-Tocopherol	0.1 mg.
2-Methyl-1,4-naphthoquinone	0.1 mg.

The animals were bedded in wheat straw and received iodized salt *ad. libitum.* Once a week 50 mg. of crystalline L-ascorbic acid, dissolved in water immediately before use, were administered by mouth. This amount is well above the minimum weekly dose recommended by Zilva (1941) and protected the animals completely from scurvy. The typical deficiency syndrome regularly developed on this diet. Oleson and coworkers (1947) used a pelleted diet, consisting of the following:

TABLE VI

Oleson et al. Diet

Ground wheat	20	lbs.
Ground whole yellow corn	15	"
Feeding oat meal	20	"
Wheat bran	5	"
Soybean oil meal (41 % expeller)	20	"
Linseed oil meal	5	"
Fish meal (60 % vacuum)	2	"
Alfalfa meal (dehydrated 20 %)	10	"
Bone meal (steamed)	1	"
Limestone (feeding)	1	"
NaCl	0.25	"
Delsterol (900,000 I.U. vitamin D)	0.125	"
Ascorbic acid	12 g.	

Each animal received 20 mg. of ascorbic acid orally three times a week as additional ascorbic acid. Signs of wriststiffness appeared much faster on this diet, and were evident in 1 to 3 weeks. Dasler and Bauer (1949) were able to produce wriststiffness in guinea pigs by feeding two different commercial, pelleted rations. They came to the conclusion that the mere production of wriststiffness is not necessarily an indication of the deficiency disease as originally described. Petering and coworkers (1948) used a diet of the following composition:

TABLE VII

Petering et al. Diet

Casein (Labco)	15.0 parts
Cornstarch	67.7 "
Brewers' yeast (Fleischmann's 50 B)	10.0 "
Salts (according to Mackenzie *et al.*, 1939)	6.0 "
Cottonseed oil (containing oil-soluble vitamins)*	1.3 "
Ascorbic acid	60 mg./week

* Supplements per 100 g. of diet: Vitamin A—1500 I.U.; α-tocopherol—4.29 mg.; Vitamin D_2—400 I.U.

This diet was very satisfactory both for the demonstration of the wriststiffness and for the assay of active fractions.

FAILURES OF OTHER WORKERS TO DEMONSTRATE THE DEFICIENCY SYNDROME

Communications from other laboratories (Homburger and Reed, 1945; Kon *et al.*, 1946) have appeared, in which the investigators reported their failure to obtain the wriststiffness condition in the experimental animals when maintained on the liquid milk diet. It should be pointed out that in these two cases the authors were not able to maintain the animals on the deficient diet for a period longer than two months. The first deficiency symptom, wriststiffness, appears usually after the animals have been kept on the deficient regime for more than two months. Both groups report a high mortality after the animals have been transferred to the milk diet. This is a general difficulty encountered by many workers. It has been our experience that only a gradual transfer to the milk diet will prevent a high mortality rate. The general practice in our laboratories was to train the animals gradually by pipette feeding with the milk mixture over a period of at least three weeks, in the meantime keeping them supplied with grains and greens. Only after they were thoroughly accustomed to the milk diet were the grains removed, and after a week the greens. In this way we never had a high mortality. Caldwell (1948), using the milk diet, could establish the deficiency syndrome in this manner

III. PHYSIOLOGICAL ASPECTS OF THE DEFICIENCY SYNDROME

1. Pathological Changes during the Deficiency

Prolonged deficiency in the antistiffness factor affects guinea pigs profoundly in a variety of ways. Though growth is retarded and stiffness develops, these are by no means all the obvious changes which occur. The purpose here is to list these deviations from normality in so far as they have been observed. (Unpublished data by R. Wulzen and A. B. Plympton.)

Guinea pigs were subjected to the standard diet of milk powder in water, salts, and vitamins (see Tables III and IV). The wheat straw used as bedding in this case was not autoclaved, which may account for the fact that the results here described were chronic rather than acute. The nondeficient, stock animals were fed rolled barley and an abundant supply of fresh, green feed daily: kale, grass, and clover when possible.

a. Growth. Weight data were gathered for 27 stock and 65 deficient animals over a period of 26 months. These values for the males of the two groups are shown graphically in Fig. 4. Comparison of numerical averages of all animals was limited to the first 14 months because of loss by death among deficient animals after that time.

For stock animals, weights summarized at 6 months, 10 months, and 14 months after the start of the experiment gave the following averages respectively: 864 g., 1037 g., and 1070 g. Comparing these values with the average weights of the deficient group, it was found that 67% of the deficients were below average normal weight of stock animals at 6 months; 83% at 10 months; 90% at 14 months.

Five control animals had been kept for 9 months on the deficient diet and were then given, in addition to this, 8 to 16 cc. cane molasses per animal per day, to supply adequate amounts of the antistiffness factor. Two animals, male and female, from this group were compared with two animals, male and female, from the deficient group. These animals had come in the same consignments, and had lived in the same pens. Very quickly the two molasses-fed animals outdistanced the deficient guinea pigs in weight and have shown steady gain up to the present time. In contrast, the two deficient animals died after 14 and 18 months with pronounced deficiency symptoms (Figs. 5 and 6).

b. Stiffness. Deficiency in the antistiffness factor produces muscular stiffness in all parts of the body, but for test purposes the forelegs proved most useful. The investigator forcibly extends the leg close to the body and applies upward pressure to the metacarpal bones on the medial side.

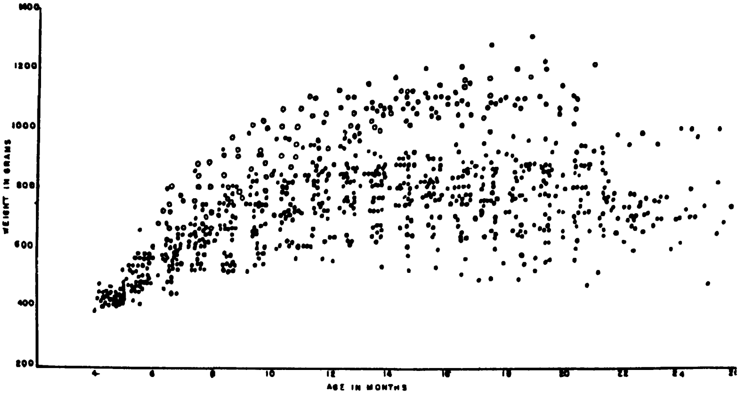


FIG. 4. Growth data on deficient and stock male guinea pigs. Solid dots indicate weights of 37 deficient males as they advanced from 4 months of age to 26 months. Circles indicate weights of 18 stock males over a corresponding, but somewhat shorter, period. Note: (1) immediate divergence of the two groups, (2) absence of overlap after 12 months of age.

In the normal animal, the paw easily bends to a position at right angles to the forearm. When stiffness has been completely developed, the wrist cannot be bent at all. All gradations between these extremes can be observed. If the paw failed to bend 45°, stiffness was considered sufficient to be called abnormal. Stock animals, to be maintained in the limber condition, must receive abundant green feed of the correct varieties. Difficulty in securing suitable green feed at certain seasons caused 20% of the stock animals to show some degree of abnormal stiffness. In contrast, 95.4% of animals fed 14 months on deficient diet showed abnormal stiffness.

The group of control animals described under the previous section on growth were also used to demonstrate the effect of the antistiffness factor

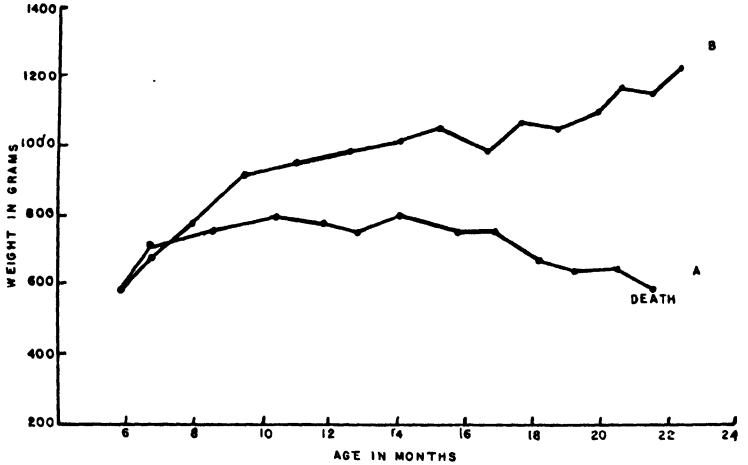


Fig. 5. Growth curves of two comparable males: A, on deficient diet 18 months, till death, B, on deficient diet plus molasses, 8–16 cc. per day. Curve could be extended beyond limits of graph; animal still alive.

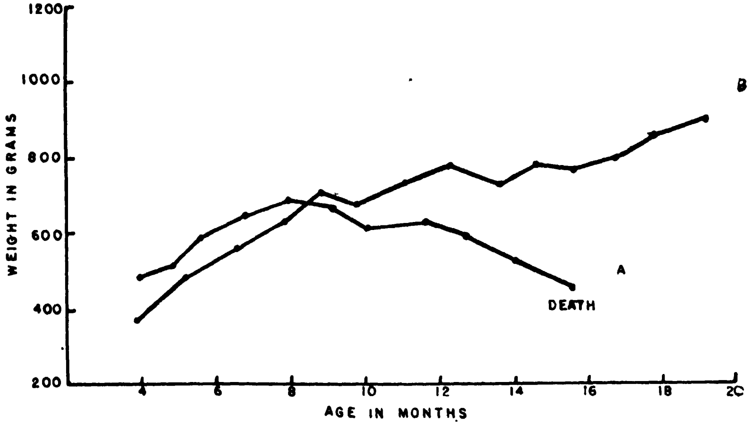


Fig. 6. Growth curves of two comparable females: A, on deficient diet 14 months, till death, B, on deficient diet plus molasses, 8–16 cc. per day. Curve could be extended beyond limits of graph; animal still alive.

on stiffness. After 9 months on the deficient diet, two animals had wrists so stiff as to be almost immobile; but after addition of molasses, 8–16 cc. per day, for 11 months, the wrists could be bent almost to the right-angle position, much beyond 45°. The remaining three, after 9 months on the deficient diet, had wrists that could be bent somewhat, though not to the 45° position; but at the end of the eleven-month treatment with molasses,

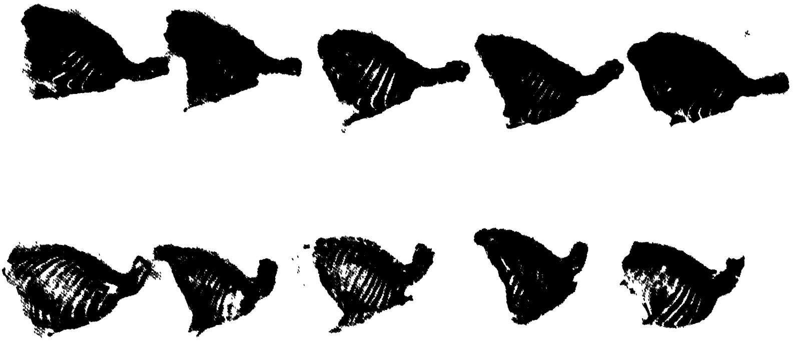


FIG. 7. Thoraces and necks of guinea pigs (dessicated). Upper row: animals on stock diet, Lower row: animals on deficient diet. Note: (a) sharp upward curve of neck in deficient animals as compared with stock, (b) humping of back in deficient group.

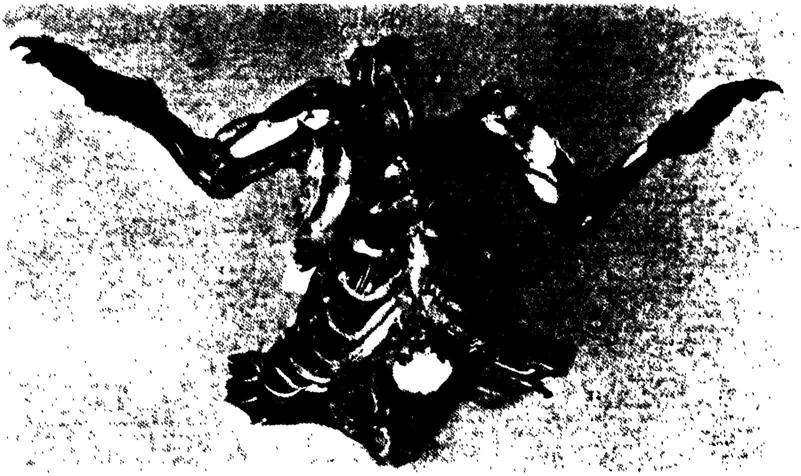
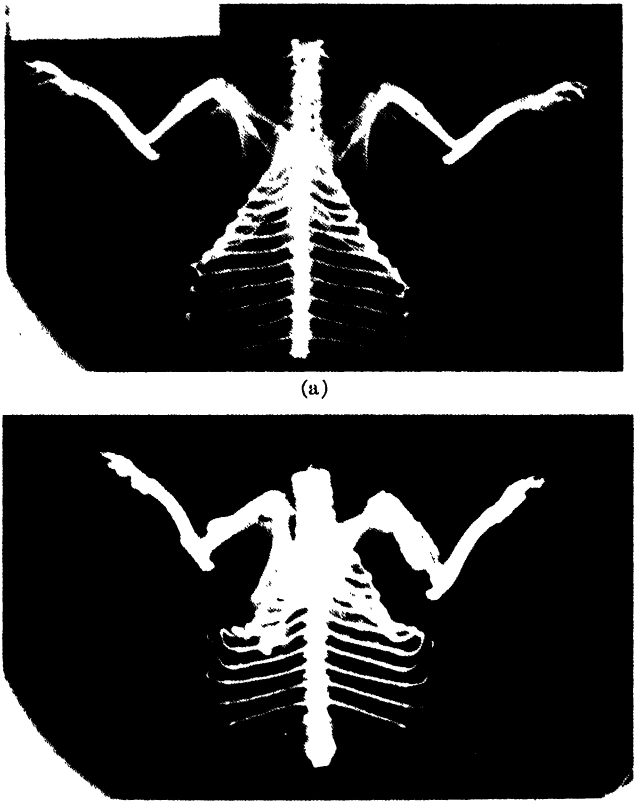


FIG. 8. Thorax and forelegs of guinea pig (dessicated). Note: (a) flaring of rib cartilages, (b) calcified ziphoid process, areas of calcification on ribs and in abscesses on forelegs.

they were entirely limber. It should be noted that we have never known animals with stiffness as severe as this to recover spontaneously.

c. Body Contour. As the deficiency advances, the neck assumes a sharp curve with the concavity upward. This position, which is maintained after death, shows plainly in Fig. 7. Such an abnormal position of the neck affects the head, which is tilted upward with elevation of the chin, the occiput being drawn toward the shoulder blades. After 14 months on the deficient diet, 87% of deficient animals showed abnormal curvature of the neck. In contrast, only 7% of stock animals showed the slightest degree of such abnormality.

Another development of advancing deficiency is dorsal curvature of the spinal column. This results in a humped back, which can easily be seen in advanced cases and can be felt in all by running the fingers along the back. At 14 months, 65% of the deficient animals were thus deformed, as against 0% of the stock animals.


(a)

(b)

FIG. 9. X-rays of guinea pig thoraces (dessicated). Upper: animal fed stock diet, Lower: animal fed deficient diet. Note: (a) flaring of rib cartilages in deficient animal as compared with smooth outline in stock animal, (b) calcification of abscesses on forelegs.

The earliest deformation in body contour noticed is a flaring of the rib cartilages in such a way that a rounded elevation can be plainly felt on each side of the thorax, ventrally. This is apparently accompanied by hardening and constriction of the bony rib case. After 14 months, 93% of deficient animals showed definite flaring of the rib cartilages (Figs. 8 and 9). None of the stock animals showed these symptoms.

d. Calcification. The deficiency syndrome is characterized by deposits of calcium phosphate scattered widely throughout the body.

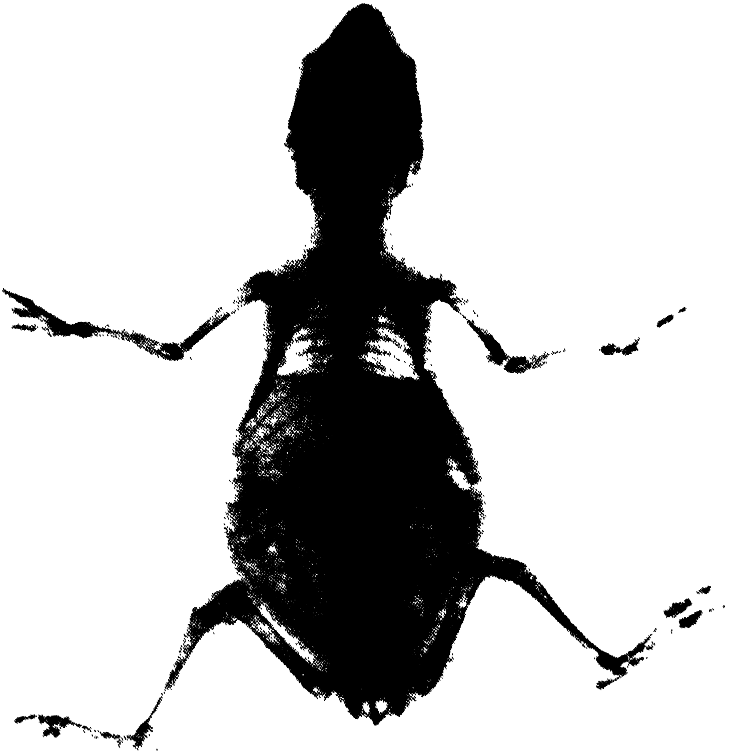


FIG. 10. Guinea pig B/79. Received the deficient diet for 105 days. Note calcium deposits around elbows, knees and feet.

The animals showed various degrees of calcification. Usually in extremely deficient animals the deposits were very extensive; a very few of the animals that had been kept on the deficient diet for long periods (more than a year) failed to show signs of calcification, although the wriststiffness symptom was severe. However, when calcium deposits do appear, they rapidly increase in size (Figs. 10–13, especially Figs. 10 and 11).

It is not surprising that the insoluble calcium deposits do not decrease during a short curative treatment of 5 days with the antistiffness factor, although the inorganic phosphorus and calcium concentrations attain normal levels during such a cure. However, a prolonged administration of large doses of the antistiffness factor eventually resulted in a resorption of even large deposits from the tissues (Figs. 14 and 15). Calcium deposits on the thoracic wall, around elbows and knees, and in the feet,

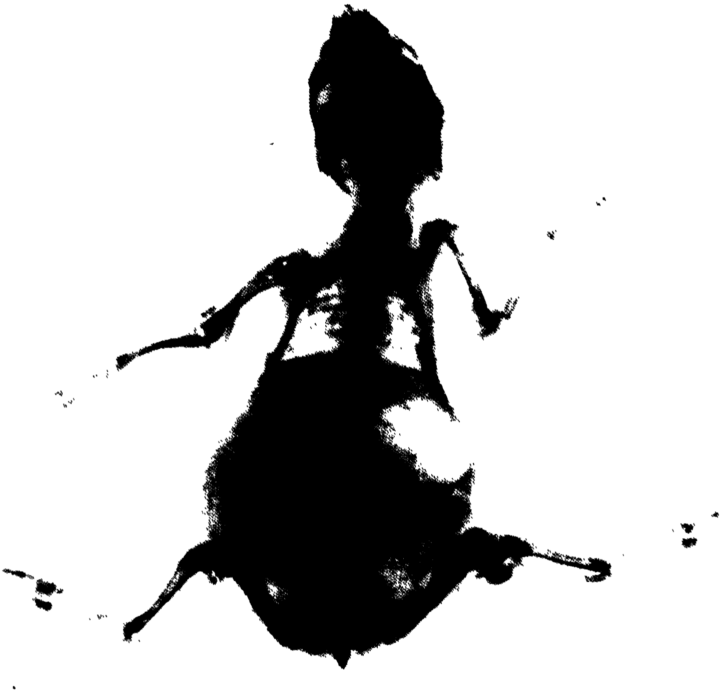


FIG. 11. Same Guinea pig (B/79), but 42 days later. Note the increased size of the deposits, which is very evident in the right elbow and left knee.

have been observed by touch, by sight, and by X-ray as they gradually disappeared.

Calcification of the muscularis of the stomach or intestine or both was often present. In the stomach, calcification was most common near the greater curvature in the cardia and fundus (Fig. 16), and usually consisted of multiple discrete small deposits that rarely caused much thickening of the wall. The area involved in some animals was quite extensive. In the colon the deposits were larger and caused appreciable thickening of the wall, but rarely involved a segment longer than 1.5 cm. (Figs. 16 and 17). Calcification was most common in the stomach and in the sharp loop of the colon, and least common in the small intestine.

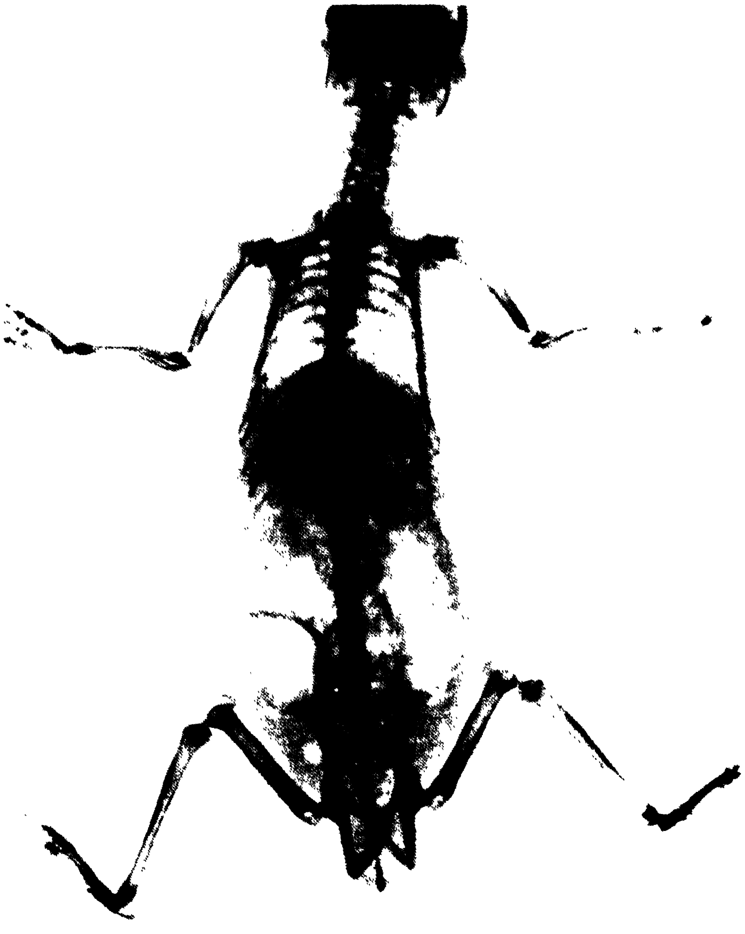


FIG. 12. Guinea pig R/85. Received deficient diet 190 days. Note more diffuse calcium deposition in the body wall.

The condition of the aorta is especially noteworthy. From the semilunar valves, through the arch, down the descending aorta, and into the femoral arteries the deposits of calcium may be easily observed and felt. The pulmonary arteries may be similarly affected (Figs. 18 and 19).

Microscopically the deposits were seen to be of irregular size and shape. In a few instances, calcification of individual muscle cells was seen, but in general the masses appeared to have been deposited between the muscle cells, and not infrequently small groups of muscle cells extended about and between the deposits. Associated with the deposits was a variable amount of collagenous tissue. Slight calcification in the muscularis mucosae was also sometimes present, but there was no calcification of the mucosa or submucosa.

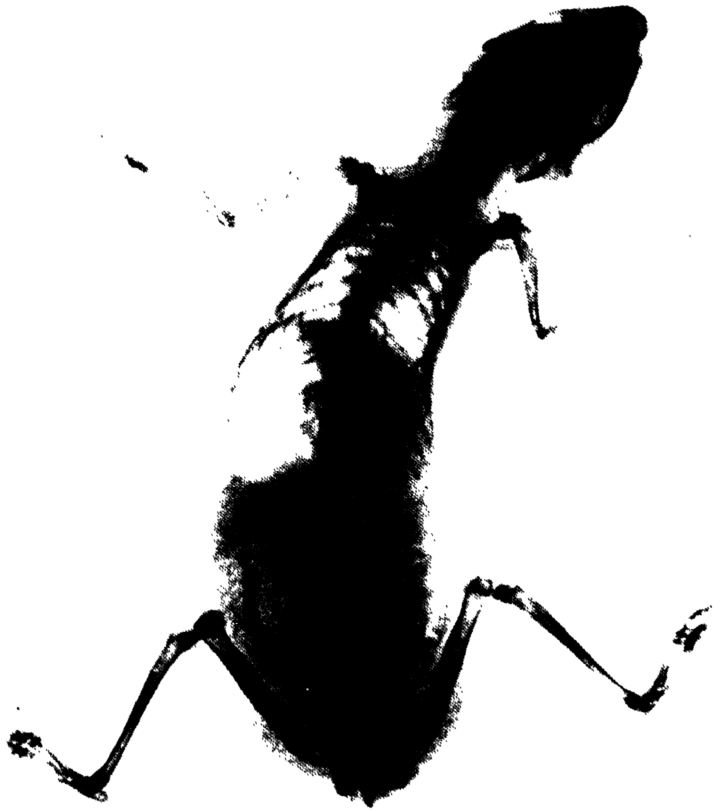


FIG. 13. Guinea pig R/79. Received deficient diet 304 days. Note large deposits in the foot pads

e. Teeth and Jaw. Abnormalities in tooth growth are common in deficient guinea pigs. Often these malformations develop gradually to a climax before death. At 14 months, deficient females showed decrease in length of upper incisors as compared to stock animals. Deficient animals of both sexes showed increase in length of lower incisors as compared to stock animals.

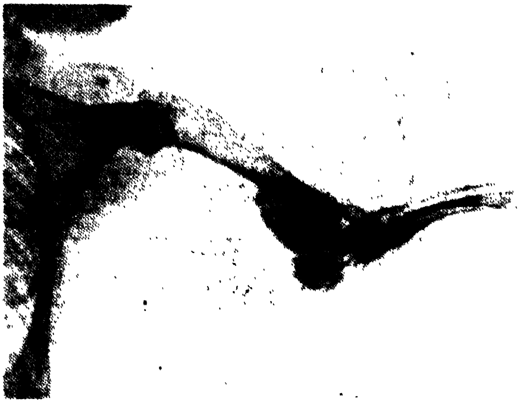


FIG. 14. Guinea pig with severe elbow stiffness resulting from 5 months on deficient diet, Table III, had been given supplement of antistiffness factor concentrated from molasses for a month when this X-ray was taken. It shows calcification around elbow joint.

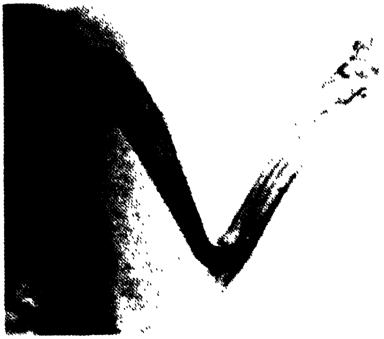


FIG. 15. The same guinea pig as in Fig. 14 after four more months' treatment with antistiffness factor. X-ray shows entire disappearance of calcified masses at elbow joint.

Following this condition, a certain sequence of changes in the incisor teeth was noted. As the lower incisors gradually elongated, they impinged upon and ground away the uppers until the lowers sometimes overlapped the uppers anteriorly. The upper incisors, thus relieved of pressure, then grew to abnormal length and curved posteriorly. The teeth were also broader and more ivory-colored than in the normal.

Lengths of both upper and lower incisors are obtained far beyond anything seen in the stock animals.

These changes were studied by viewing the teeth in profile. After 14 months, 20% of the deficient animals showed very abnormal profile

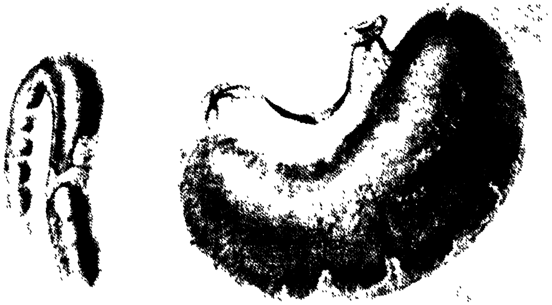


FIG. 16. At left: Sharp loop of colon from deficient guinea pig showing area of solid calcification. At right: Stomach from deficient guinea pig showing granular calcification in region of fundus and greater curvature.

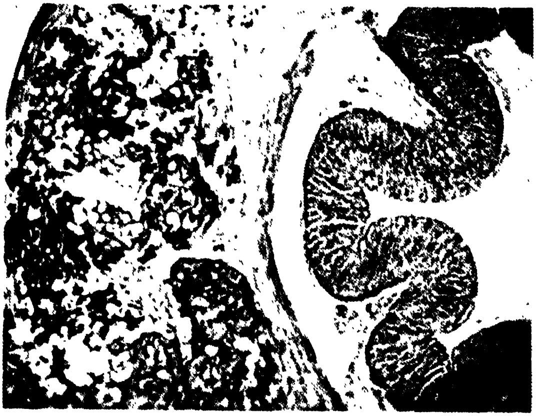


FIG. 17. Transverse section through sharp loop of colon from guinea pig deficient in antistiffness factor. At right, lumen of colon; at left, masses of calcification in outer layers of wall.

of the upper incisors, and 24% additional animals showed slightly abnormal profile. None of the stock animals had this deformation of the teeth. (Fig. 20.)

Corresponding abnormalities occur in the molars but were studied only at autopsy. In extreme cases the lower molars grew medially and completely closed over the tongue anteriorly. The upper molars flared outward, cutting into the cheek.

The stiffening of the jaw muscles, probably accompanied by similar changes in swallowing muscles, the malformation of the neck, the deformation of the teeth all render the taking of food extremely difficult, even with pipette feeding, and in the end impossible. Starvation appears to be a common cause of death among these deficient animals.

f. Hearing. The well-known ear-flick reflex of guinea pigs was used to determine hearing ability. When well developed, it is a folding back of the ear against the side of the head in response to auditory stimuli; in diminished form, it is a mere quiver of the edge of the ear. An audio-signal generator and loud speaker furnished signals varying from 0 to 17,000 cycles per second. Both stock and deficient animals were subjected to frequent hearing tests over a period of 7 months. The 68 nondeficient, stock animals showed normal range of ear-flick response (150–16,000 c.p.s., approx.) at each test over the 7 month period, the most restricted range being 2,000–10,000 c.p.s. The 68 deficient

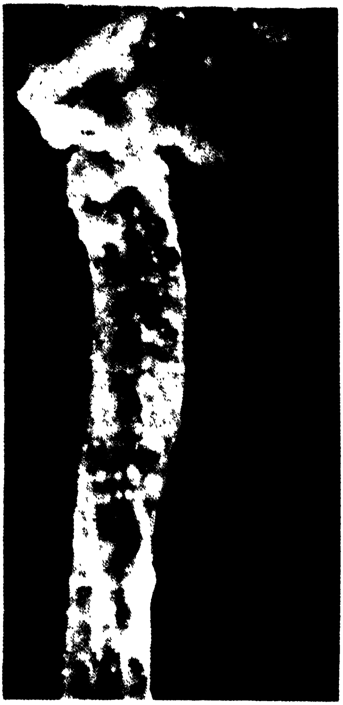


FIG. 18. Aorta slit lengthwise and laid open. Note lines of small nodules on intima. Calcium deposits show here and there.

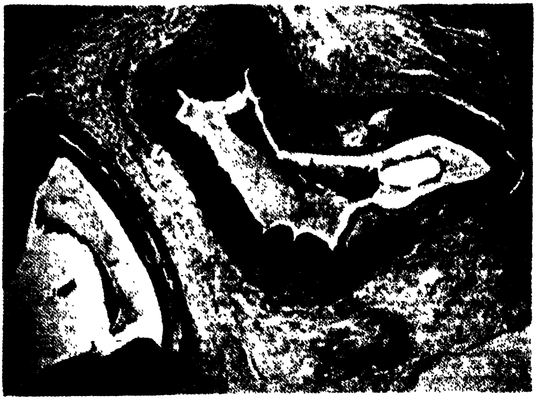


FIG. 19. Transverse section through aorta and pulmonary artery from guinea pig deficient in antistiffness factor. Note calcification in the media.

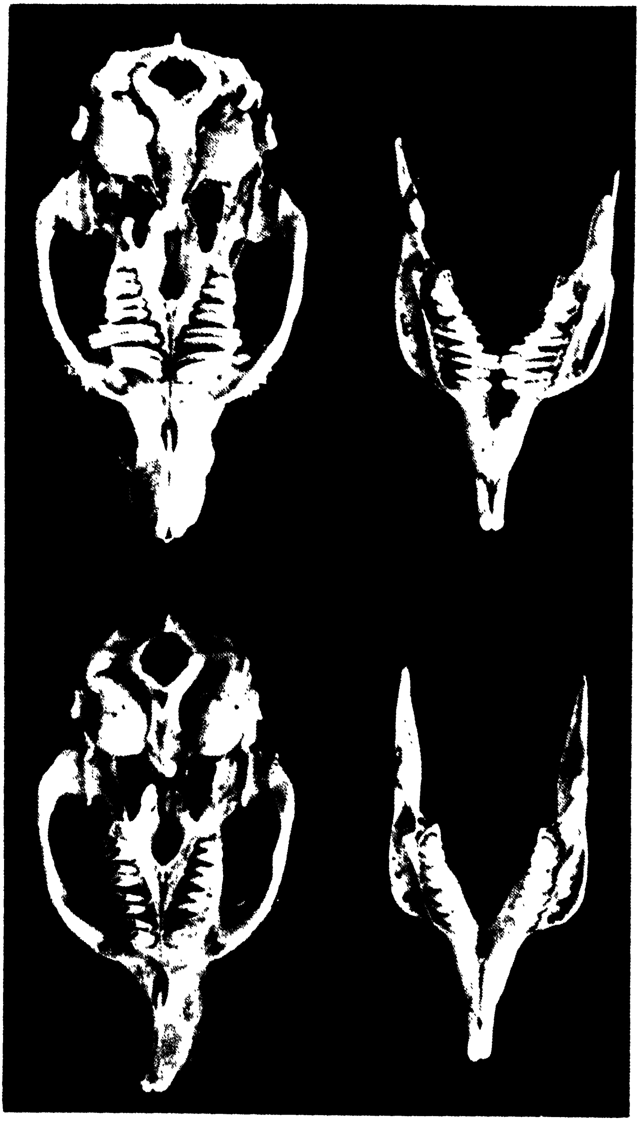


FIG. 20. Above, upper and lower jaws of guinea pig in advanced stage of deficiency. Below, upper and lower jaws of normal guinea pig. Notice overgrowth of molar teeth in deficient animal.

animals tested had been on the deficient diet for at least 11 months before the hearing tests started. Eleven of these gave no ear-flick response at the first trial and never did give any hearing response during the 7-month testing period. Eight more had become deaf, that is, gave no ear-flick response, by the end of the seven-month testing period, making a total of 19 deaf animals. Thus, at the conclusion of the experiment, 28% of the deficient animals were deaf, according to the ear-flick test, in contrast to 0% among the stock animals.

Statistical analysis showed that animals fed stock diet had significantly wider range of response to audio signals than those fed deficient diet (F 18.34 with 1 and 118 degrees of freedom), (Wulzen and Plympton, 1949).

g. Summary of Deficiency Symptoms. Abnormalities in growth, stiffness body contour, calcification, tooth growth, and hearing were tabulated on a scale of 10 with the following results. The average number of these abnormalities per deficient animals was 6.2, as contrasted with 1.6 abnormalities per stock animal and 0.6 per control animal. The control animals were five in number and were fed molasses in addition to the deficient diet (see under "Growth," Section III, 1, *a*).

h. Blood Pressure. The mean arterial occlusion pressure of 53 animals on the deficient diet for 8 months and over was 42.45 mm.; of 42 stock animals, 29.7 mm. The difference in mean value was 14 times the probably error and hence was statistically significant. Therefore, the fully developed deficiency results in rise of blood pressure (Bahrs and Wulzen, 1947).

i. Blood Picture. The hemoglobin value for 66 guinea pigs deficient in the antistiffness factor was 69% that of the stock animals. The erythrocyte count was 4,964,000 for the stock and 3,935,000 for the deficients. The average erythrocyte diameter for stock animals was 7.35 and for deficient animals 7.69 μ, a statistically significant change. The anemia was typically normochromic, hypocythemic, and normocytic or macrocytic in contrast to the hypochromic, microcytic picture found in iron anemia. In general, the more severe the hypocythemia, the greater was the macrocytosis. There were no cases of microcytic anemia.

The erythrocytic sedimentation, determined by the Landau-Adams microsedimentation method, showed a statistically significant increase over the normal values for guinea pigs. In the young guinea pigs the first to show elevated sedimentation rates were also the first to show wriststiffness. Eighty per cent of the guinea pigs 17 to 39 weeks on the skim milk diet had abnormally high sedimentation rates. Only 60% of those on the diet 45 to 81 weeks had abnormal values, probably due to the death of the more susceptible animals. The average microsedimentation value for stock animals was 2.24 mm. and for deficient animals 5.12 mm.

The five deficient guinea pigs fed a supplement of molasses (see "Growth" Section III, 1, *a*) were normal with reference to red cell diameter, red cell count, and sedimentation rate. The hemoglobin had returned to 85% of normal. However, hemoglobin formation is frequently slower than erythrocyte formation in recovery from anemia.

There was a moderate to severe leucocytosis. The average white cell count for 52 deficient animals was 16,690 but there were a few counts as high as 30,000 and 40,000. The white cell count for 55 stock animals averaged 7,500.

The differential counts of 85 deficient animals showed a granulocytosis with neutrophilia, eosinophilia, or both. There were significant differences between the differential counts of animals with moderate wriststiffness and animals with severe wriststiffness.

Relatively stiff animals fed the deficient diet for periods ranging frcm 24 to 110 weeks showed a persistent eosinophilia which generally rose with increasing stiffness. In every case eosinophilia was associated with moderate or severe stiffness. In only 2 cases out of 85 was severe stiffness not accompanied by eosinophilia. The severely stiff females had an eosinophilia of 16.5% compared to the severely stiff males with an average eosinophilia of 6% and the stock average of 2%.

The five deficient guinea pigs fed a supplement of molasses (see "Growth" Section III, 1, *a*) had normal white cell counts (Weimar and Wulzen, 1948, 1950).

j. Thirst. When deficiency is well established, the affected animals drink excessive amounts of water.

k. Diarrhea. This condition develops in every case where the disintegration process is prolonged. Feces have a characteristic creamy consistency and a light brown color. Often the depletion caused by this diarrhea is a primary cause of death.

l. Loss of Hair. Very deficient animals regularly have rough and thinning hair. They sometimes become almost denuded.

m. Disabled Legs. When the deficiency is well developed, animals are inclined to lie on the side to relieve painful limbs and may drag the hind legs when walking.

n. Mortality. Death from the deficiency seems most often to be due to persistent diarrhea and to jaw and tooth conditions which lead to starvation. Deaths begin at about 10 months after starting on the diet, with highest incidence at 16 and 17 months. Of 65 deficient animals, 43 died with plainly marked deficiency symptoms in the course of about 24 months. Among the stock animals, in a corresponding time, only two or three were lost, due to pregnancy difficulties. In a simultaneously run control group of five animals given molasses in addition to the deficient diet (see under "Growth" Section III, 1, *a*), all were in fine condition at the time of writing. Of the 22 living deficient animals, all show stiffness and all except 4 (females), subnormal weight. Deficiency symptoms are so developed in these animals so as to make it appear inevitable that most of them will soon die.

o. Alleviation of Symptoms. Stiffness caused by deficiency has been cured many hundreds of times (Section IV, 2) by various substances containing the antistiffness factor, such as raw cream, molasses, and other sugar-cane products, and green vegetables. Even maximum stiffness has been cured by sufficiently prolonged treatment with molasses or raw cream (see Figs. 21–23).

p. Pathological Findings upon Autopsy. The lesions found during the deficiency consisted essentially of abscess formation, necrosis and calcification. The organs most commonly affected were the cardiovascular system, the gastrointestinal tract, liver, kidneys, skeletal muscle, footpads, and periarticular tissues (Harris and Wulzen, 1949a and in press).

In the musculoskeletal system, abscesses often developed in the footpads. Ulceration over the abscesses occurred sometimes, but destruction of bones of the feet was never encountered. Abscesses were also found about the knees, elbows, and shoulders, intramuscularly at the tip of he scapula, in the intercostal muscles and over the ribs laterally and dorsally, and deep within the muscles of the legs adjacent to the periosteum of the femur or humerus. The abscesses at first contained an opalescent, glairy white fluid that subsequently became inspissated and opaque, and ultimately underwent calcification. The abscesses about the shoulders, elbows, and knees were usually associated with proliferation of collagenous tissue about the joints, and periostial new bone formation of the long bones near the affected joints. Usually the joints were not invaded, but were occasionally filled with pus, and when this occurred the articular cartilage was rarely eroded.

Necrosis of skeletal muscle varied from necrosis of scattered fibers to involvement of the majority of the fibers in some muscles. The earliest time at which muscle necrosis could be discovered was around two months. Slightly injured muscles appeared grossly normal, but more severe injury was manifested by slight pallor and by the presence of opaque, grayish-yellow, longitudinal streaks of various lengths and breadths. Occasionally, some of the streaks were coarse and beaded. In some animals these streaks were seen best or exclusively in the muscles of the chest wall, notably the serratus anterior and pectoral muscles, and the muscles

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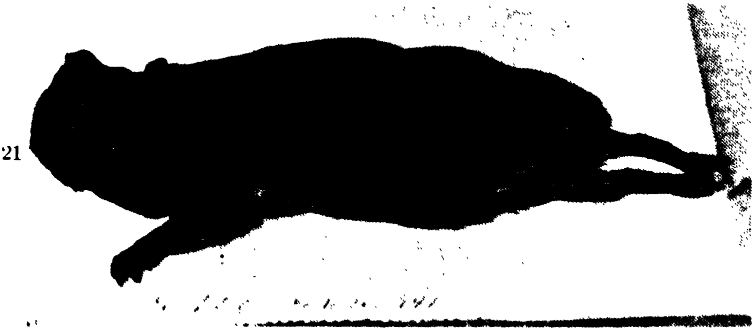

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23

FIGS. 21, 22, 23. Showing stages of recovery from deficiency in antistiffness factor through use of raw cream.

FIG. 21. Prostrate animal: limbs too stiff to bend without breaking.

FIG. 22. Able to balance when placed upright, but still too stiff to move. Two-week interval between Figs. 21 and 22.

FIG. 23. Condition normal. Three-week interval between Figs. 22 and 23.

attached to the scapula. In others they were clearly evident in the abdominal muscles and muscles of the leg also.

Microscopically there was necrosis with hyalinization, and in some cases with regeneration of muscle fibers. In some instances injury was manifested by slight to great increase in nuclei in some muscle fibers. More commonly, however, the changes were predominantly degenerative and characterized by the presence of fragmented muscle fibers with swollen, hyaline, eosiniphilic, curdy cytoplasm and loss of nuclei. Often an otherwise normal fiber was fractured and the terminal portion had the appearance just described. Some fibers had lost their nuclei, but the transverse striations were maintained and even accentuated. Still others showed loss of longitudinal striation. Some fibers were invaded by macrophages, and in places these were aggregated to form foreign body giant cells associated with the fragmented cytoplasm. Frequently although muscles were the seat of much necrosis, few or no macrophages were present. At times there was calcification, slight or fairly extensive, of the necrotic muscle cytoplasm, and this was accompanied by macrophage infiltration with or without giant cell formation. In a few instances, the sole evidence of muscle injury was the presence of macrophages or giant cells in scattered fibers, with or without calcification. In the more severely injured muscles entire fields consisted solely of necrotic fibers, and in the less injured ones, necrotic and viable fibers intermingled in various proportions. In the muscles that were described as containing beaded streaks there were nodules of more extensive calcification; it appeared probable that at least some of these had their origin in small abscesses.

The liver contained scattered, opaque, minute yellow dots or delicate yellow streaks, and in a few instances, subcapsular foci of necrosis a few millimeters in width. Microscopically the dots and streaks were seen to be due to necrosis of liver cells. Rarely was there any leukocytic reaction, and when present it was slight. Calcification of the necrotic cells was often observed.

The kidneys were usually pale, granular, somewhat enlarged and heavier than normal, both absolutely and in proportion to body weight. Calcified masses of irregular size and shape were seen in convoluted and collecting tubules, and at times foci of calcification of the interstitial tissue were seen in both cortex and medulla. Some of the masses obviously obstructed the tubules in which they lay. Calcification of the walls of the arteries was seen very seldom, and in these cases the lumen was not reduced. The glomerulus was reduced in size.

Testicular atrophy was often observed, ranging from cessation of spermatogenesis in some tubules to complete loss of spermatogonia from all tubules.

2. Physiological and Biochemical Changes during the Deficiency

a. Phosphorus Metabolism. The abnormal deposition of calcium phosphate during the deficiency is suggestive of a derangement of the phosphorus metabolism, which would be reflected in an abnormal distribution of the acid soluble phosphorus fractions in the liver, kidneys, and blood of deficient guinea pigs. That this was indeed the case is shown in Table VIII (van Wagtendonk, 1944), which represents the mean concentrations of the total acid-soluble phosphorus and of the fractions in the liver of guinea pigs receiving (a) the stock diet, (b) the deficient skim milk diet and (c) the deficient diet supplemented with the antistiffness factor. The most significant change took place in the easily hydrolyzable phosphorus. This fraction showed a decrease of close to 50% after the animals had received the deficient diet for one week. The lower limit of this fraction appeared to be 3.4 mg. per 100 g. of liver and was reached after a year. The total acid-soluble P fluctuated during the

TABLE VIII

Distribution of Acid-Soluble Phosphorus in Liver of Normal and Deficient Guinea Pigs

				Mean and s.e., mg. per 100 g.						
Age *wks.*	Diet	Time on diet *wks.*	No. of determinations	Total acid-soluble P	Inorganic P	Easily hydrolyzable P	Alcohol-insoluble P	Mercuric-insoluble P	Alcohol-soluble P	Sum of fractions
14	Stock	14	15	111.9 ±2.0	15.4 ±0.7	14.2 ±0.9	24.9 ±0.5	46.6 ±0.7	12.8 ±0.7	113.9 ±2.2
65	Stock	65	9	105.5 ±2.0	18.9 ±0.8	16.6 ±2.0	17.9 ±0.8	45.5 ±0.7	10.6 ±1.0	109.5 ±2.2
14	Deficient	1	15	103.8 ±0.8	27.4 ±0.9	7.9 ±0.1	20.5 ±0.8	40.4 ±0.5	9.7 ±0.5	106.5 ±0.6
15	Deficient	2	15	122.0 ±2.7	26.6 ±1.8	5.4 ±0.7	25.9 ±0.9	44.4 ±1.5	16.4 ±0.7	118.8 ±2.8
16	Deficient	3	11	128.9 ±2.8	25.8 ±0.7	6.1 ±0.6	23.9 ±0.9	52.1 ±2.4	20.9 ±0.4	123.4 ±0.4
20	Deficient	7	8	118.9 ±4.0	24.6 ±1.2	4.9 ±0.6	19.3 ±1.9	55.1 ±2.0	15.4 ±0.9	119.4 ±3.7
41	Deficient	28	11	131.6 ±1.9	30.7 ±1.5	4.0 ±0.2	20.5 ±0.5	56.4 ±0.7	18.0 ±0.5	129.6 ±1.9
70	Deficient	57	16	165.6 ±1.5	36.6 ±0.9	3.4 ±0.3	19.3 ±0.4	79.5 ±0.6	26.3 ±0.4	165.1 ±1.4
17	Deficient + a.s.f.*	4	14	110.5 ±1.2	21.7 ±0.9	12.7 ±0.9	22.3 ±0.6	42.8 ±1.2	10.4 ±0.6	109.9 ±1.3
23	Deficient + a.s.f.*	10	14	112.6 ±2.4	24.8 ±1.0	14.2 ±0.9	19.1 ±1.1	43.3 ±0.6	10.9 ±1.1	111.3 ±2.7

* 1000 units of the antistiffness factor every other day during the whole course of the experiment.

first weeks of the deficiency. These fluctuations can be accounted for by the changes occurring in the other fractions. During the later stages the total acid-soluble P showed a significant increase which was caused by the increases in the concentrations of the inorganic P, the mercuric-insoluble P, and the alcohol-soluble P. If the deficient skim milk diet was supplemented with the antistiffness factor during the whole course of the experiment, the distribution of the acid-soluble P was essentially normal, with the exception of the high concentration of the inorganic P fraction.

The distribution of the acid-soluble P in the kidneys during the deficiency (Table IX) followed the same trend as that in the liver. There is however a much larger increase in the concentration of the inorganic P fraction.

TABLE IX

Distribution of Acid-Soluble Phosphorus in Kidneys of Normal and Deficient Guinea Pigs

Age *wks.*	Diet	Time on diet *wks.*	No. of determinations	Mean and S.E., mg. per 100 g.						
				Total acid-soluble P	Inorganic P	Easily hydrolyzable P	Alcohol-insoluble P	Mercuric-insoluble P	Alcohol-soluble P	Sum of fractions
14	Stock	14	15	95.2 ±0.2	15.1 ±0.1	14.0 ±0.3	15.6 ±0.1	45.2 ±0.6	12.6 ±0.9	102.5 ±1.7
65	Stock	65	9	100.4 ±2.0	26.1 ±1.2	10.6 ±0.5	17.9 ±0.9	27.9 ±1.0	11.2 ±0.2	93.7 ±0.9
14	Deficient	1	15	95.8 ±1.0	26.6 ±0.1	7.8 ±0.1	8.8 ±0.1	41.8 ±0.1	15.1 ±0.1	100.1 ±0.9
15	Deficient	2	15	107.8 ±0.9	27.9 ±0.1	3.1 ±0.1	15.9 ±0.1	41.4 ±1.1	16.5 ±0.1	104.8 ±1.9
16	Deficient	3	11	117.3 ±0.1	30.2 ±0.1	3.6 ±0.1	24.4 ±0.1	40.2 ±0.1	16.3 ±0.1	114.7 ±1.3
20	Deficient	7	8	112.6 ±2.9	32.6 ±1.0	3.3 ±0.1	20.1 ±0.7	40.6 ±0.3	16.1 ±0.9	112.7 ±1.9
41	Deficient	28	11	129.3 ±3.5	50.0 ±2.5	3.6 ±0.3	16.8 ±0.2	44.7 ±1.0	11.3 ±0.2	126.4 ±3.0
70	Deficient	57	16	153.2 ±2.2	65.3 ±2.0	2.3 ±0.2	17.6 ±0.2	43.4 ±0.9	19.2 ±0.4	147.8 ±2.9
17	Deficient + a.s.f.*	4	14	112.0 ±0.7	27.1 ±0.6	11.9 ±0.4	16.6 ±0.6	43.4 ±0.9	14.2 ±0.4	113.2 ±0.4
23	Deficient + a.s.f.*	10	14	103.9 ±0.8	26.2 ±0.7	10.8 ±0.1	15.7 ±0.1	39.9 ±0.1	11.1 ±0.1	104.2 ±1.5

* 1000 units of the antistiffness factor every other day during the whole course of the experiment.

Administration of the antistiffness factor to animals that had been on the deficient diet for some time resulted in a return to normal values of the concentration of the easily hydrolyzable P during the short-time administration of the factor in curative dosages (Table X). A comparison between the distribution of the acid-soluble P in the liver and kidneys of guinea pigs raised on a stock diet and on a raw milk diet revealed that the distribution of the acid-soluble P in the liver and kidneys of animals raised on a raw milk diet was comparable to that found in these organs of animals raised on a stock diet, with the exception of the con-

TABLE X

Effect of "Cure" with Antistiffness Factor on Distribution of Acid-Soluble Phosphorus in Liver and Kidneys of 20 Week-Old Deficient Guinea Pigs on Diet for 7 Weeks

	Diet	No. of determinations	Mean and S.E., mg. per 100 g.						
			Total acid-soluble P	Inorganic P	Easily hydrolyzable P	Alcohol-insoluble P	Mercuric-insoluble P	Alcohol-soluble P	Sum of fractions
Liver	Deficient	8	118.9	24.6	4.9	19.3	55.1	15.4	119.4
			±4.0	±1.2	±0.6	±1.9	±2.0	±0.9	±3.7
	Deficient + a.s.f.*	7	110.6	22.6	13.2	15.9	44.7	15.8	112.2
			±3.4	±1.3	±0.8	±0.7	±0.9	±0.9	±1.9
Kidneys	Deficient	8	112.6	32.6	3.3	20.1	40.6	16.1	112.7
			±2.9	±1.0	±0.1	±0.7	±0.3	±0.9	±1.9
	Deficient + a.s.f.*	7	108.1	24.0	9.3	18.5	39.7	16.3	107.8
			±2.9	±0.1	±0.5	±1.8	±0.1	±0.9	±0.6

* 1000 units of the antistiffness factor for the last 5 days.

TABLE XI

Comparison between Distribution of Acid-Soluble Phosphorus in Liver and Kidneys of 65 Week-Old Guinea Pigs on Stock Diet and Raw Milk Diet

	Diet	Time on diet *wks.*	No. of determinations	Mean and S.E., mg. per 100 g.						
				Total acid-soluble P	Inorganic P	Easily hydrolyzable P	Alcohol-insoluble P	Mercuric-insoluble P	Alcohol-soluble P	Sum of fractions
Liver	Stock	65	9	105.5	18.9	16.6	17.9	45.5	10.6	109.5
				±2.0	±0.8	±2.0	±0.8	±0.7	±1.0	±2.2
	Raw milk	52	6	112.4	22.6	11.3	18.3	43.1	14.9	110.2
				±1.8	±1.2	±0.6	±0.7	±0.8	±0.7	±1.1
Kidneys	Stock	65	9	100.4	26.1	10.6	17.9	27.9	11.2	93.7
				±2.0	±1.2	±0.5	±0.9	±1.0	±0.2	±0.9
	Raw milk	52	6	154.2	79.3	2.4	19.1	49.1	11.5	161.4
				±1.1	±1.2	±0.5	±0.9	±0.6	±0.5	±1.1

centration of the inorganic P. This fraction was considerably higher in animals which had received the raw milk diet (Table XI).

A similar decrease in the concentration of energy-rich phosphate was found in the muscle (Table XII, van Wagtendonk and Lamfrom, 1945). This decrease paralleled the drop in the concentration of the easily hydrolyzable P in the liver. The impaired function of the muscle during the deficiency of the antistiffness factor is therefore correlated with a lowered content of compounds containing energy-rich phosphate bonds. A similar decrease in the concentration of creatine-phosphate and adenosinetriphosphate was reported by Reinhold and Kingsley (1938) in drystrophic guinea pigs deficient in vitamin E. Although both a deficiency of vitamin E and a deficiency of the antistiffness factor result in a lowered concentration of creatine phosphate and adenosinetriphosphate in the muscle, a distinct difference is found in the fate of creatine. A deficiency of vitamin E is characterized by a severe urinary excretion of creatine, correlated with the degree of muscular dystrophy (Houchin and Mattill, 1942). This was demonstrated by a series of experiments where the animals were fed a diet deficient in both vitamin E and the antistiffness factor (van Wagtendonk *et al.*, 1944c). The diet was based on the one used by MacKenzie *et al.* (1941), and consisted of:

TABLE XII

Distribution of Acid-Soluble P in Muscle of Guinea Pigs Receiving Stock Diet and Skim Milk Diet

Age *wks.*	Stock diet				Deficient diet				
	No. of determinations	Mean, mg. per 100 g.			Time on diet *wks.*	No. of determinations	Mean, mg. per 100 g.		
		Inorganic P	Creatine phosphate	ATP-ADP*			Inorganic P	Creatine phosphate	ATP-ADP*
13	10	54.1	7.0	12.4	1	15	48.7	7.8	18.7
14					2	15	69.1	7.1	12.4
15					3	15	51.6	10.0	14.8
16					7	8	71.0	7.0	14.5
20	15	37.2	9.2	20.6	10	15	54.0	7.9	7.6
23					19	5	76.1	4.5	4.4
29					28	15	65.2	4.2	3.8
41					59	16	80.1	3.0	2.8
72	10	42.7	12.0	19.8					

* ATP and ADP denote respectively adenosinetriphosphate and adenosinediphosphate.

TABLE XIII

Casein (Smaco, vitamin free)	1050 g.
Dextrin (commercial cornstarch)	4830 g.
Yeast (Fleischmann's dried irradiated brewers' yeast)	700 g.
Salt mixture, as described by MacKenzie *et al.* (1941)	420 g.
Orange juice (separately each day)	1 ml./100 g. weight.

When this diet was supplied with both vitamin E and the antistiffness factor no creatine excretion was evident (Table XIV). If the diet was supplemented with the antistiffness factor only, creatinuria developed which, however, was less severe than when both the antistiffness factor and vitamin E were absent from the diet.

TABLE XIV

Day	Deficiency Indices														
	Control			Deficient diet			Deficient diet plus antistiffness factor			Deficient diet plus α-tocopherol			Deficient diet plus antistiffness factor plus α-tocopherol		
	Weight	Cr/Crtinine	Stiffness	Weight	Cr/Crtinine	Stiffness	Weight	Cr/Crtinine	Stiffness	Weight	Cr/Crtinine	Stiffness	Weight	Cr/Crtinine	Stiffness
	g.			g.			g.			g.			g.		
1	419	0.61	4-4	393	0.31	4P-3	473	0.47	4-4	446	0.51	4P-3	435	0.49	4-4
53	564	0.38	4-4	474	0.94	3-2	518	0.98	4-4	494	0.39	3-2.5	512	0.27	4-4
79	628	0.17	4-4	463	0.63	3-1.5	488[1]	1.99	4-4	497	0.84	3-2	532	0.96	4-4
86	670	0.37	4-4	475	2.91	3-1	475	1.96	4-4	497	1.03	2-1.5	531	0.76	4-4
91	687	0.45	4-4	470	3.29	2-1E	479	0.81	4-4	482	1.09	1-1E	522	0.64	4-4
97	721	0.57	4-4	460	8.30[2]	1-1E	480	3.09	4-4	513	0.65	1-1E	560	0.69	4-4

The figures in this table are the average values for the groups.

Note: $\frac{Cr}{Crtinine}$ stands for the $\frac{Creatine}{Creatinine}$ ratio.

[1] One animal of this group had died from diarrhea on the seventy-sixth day.

[2] Two animals of this group died on the 98th day from severe deficiency.

The derangement in phosphorus metabolism is also evident from the change in alkaline serum phosphatase in the blood of deficient guinea pigs (van Wagtendonk *et al.*, 1944b). A decrease in the alkaline serum phosphatase was evident from the onset of the deficiency. Administration of the antistiffness factor did not cause an immediate return to normal values, not even in dosages as much as 1000 times the dosage required to alleviate the stiffness and to normalize the abnormal distribution of the acid-soluble P in the liver and kidneys of guinea pigs deficient in the antistiffness factor (Table XV).

TABLE XV

Alkaline Serum Phosphatase of Guinea Pigs Raised on a "Stock" Diet and on a Diet Deficient in the Antistiffness Factor

Age *wks.*	Diet	Weeks on diet	No. of determ.	Mg. P. liberated per 100 cc. serum *Mean*
13	Stock	13	15	10.7
20	Stock	20	13	8.8
65	Stock	65	10	2.8
14	Skimmed milk	1	15	7.5
16	Skimmed milk	2	15	6.5
20	Skimmed milk	7	15	5.3
41	Skimmed milk	28	15	2.8
65	Skimmed milk	52	15	0.4
24	Skimmed milk[1]*	11	15	7.6
20	Skimmed milk[2]*	7	15	5.3

[1] 1000 Units of the antistiffness factor every other day during the whole course of the experiment.

[2] 1000 Units of the antistiffness factor during the last 5 days of the experiment.

* One unit was arbitrarily defined as follows: A solution of the compound in Wesson oil contains 1 unit per cc. if, when 1 cc. is administered daily for 5 consecutive days to a deficient animal, it alleviates the induced stiffness in this time.

b. Calcium and Phosphorus Metabolism. As one of the results of the deficiency of the antistiffness factor deposits of calcium phosphate may appear in many tissues, the most conspicuous deposition being in the aorta of deficient animals (see "Pathological" section, p. 83). The high concentration of inorganic phosphate in liver and kidneys coincided with a high concentration of inorganic phosphate in the blood and muscle (Table XVI, van Wagtendonk *et al.*, 1944a). Administration of

TABLE XVI

Inorganic Phosphorus and Total Calcium in the Blood and Calcium in the Muscle of Guinea Pigs Raised on a "Stock" Diet

Age in weeks	Weeks on diet	No. of determ.	Inorganic phosphorus mean and S.E.	Calcium (blood) mear and S.E.	Calcium (muscle) mean and S.E.
			mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 g.*
11	8	7	—	9.5 ± 0.2	—
13	10	5	—	9.9 ± 0.6	—
14	11	27	3.0 ± 0.3	10.8 ± 0.3	9.7 ± 1.6
65	62	11	3.3 ± 0.9	903 ± 0.5	9.0 ± 1.0

the antistiffness factor reduced the high value in muscle to normal levels while the high concentration of the inorganic phosphate in the blood was prevented by the continuous administration of the antistiffness factor along with the deficient diet. The increase in inorganic phosphorus in the blood was accompanied by an increase in the calcium concentration (Table XVII). This simultaneous rise in the concentration of calcium and inorganic phosphorus is favorable for the formation of colloidal calcium phosphate. According to McLean and Hinrichs (1938) its formation follows when the product [Ca] [P] becomes > 3 (the concentrations of both components are expressed in millimoles per liter). As can be seen from Table XVIII this product exceeded the value of 3 a week after the animals were started on the deficient diet. The colloidal calcium phosphate was rapidly removed from the blood. A higher concentration of this complex might favor precipitation of calcium phosphate in the tissues. That a colloidal form of calcium phosphate is being formed during the deficiency is supported by the finding that there is a considerable increase n the concentration of nondiffusible calcium (van Wagtendonk and Freed, 1947). The concentration of this complex attained a high plateau shortly after the animals received the deficient diet (Tables XIX and XX). A return to

TABLE XVII

The Influence of Various Doses of the Antistiffness Factor upon the Inorganic Phosphorus and the Total Calcium in the Blood and the Calcium in the Muscle of Guinea Pigs.Raised on a "Skimmed Milk" Diet

Age *wks.*	Diet	Weeks on diet	No. of determ.	Inorganic phosphorus mean and S.E.	Calcium (blood) mean and S.E.	Calcium (muscle) mean and S.E.
				mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 g.*
29	Skimmed milk	16	5	4.7 ± 0.2	12.1 ± 1.3	19.4 ± 2.6
29	Skimmed milk 0.01 μg. a.s.f. last 5 days	16	5	3.1 ± 0.2	12.2 ± 0.6	17.3 ± 1.5
29	Skimmed milk 10 μg. a.s.f. last 5 days	16	10	2.8 ± 0.1	11.7 ± 0.6	18.8 ± 1.2
24	Skimmed milk 10 μg. a.s.f. every other day during whole experiment	11	11	4.0 ± 0.2	7.9 ± 0.6	10.5 ± 1.0

TABLE XVIII

Inorganic Phosphorus and Total Calcium in the Blood and Calcium in the Muscle of Guinea Pigs Raised on a "Skimmed Milk" Diet

Age *wks.*	Weeks on diet	No. of determ.	Inorganic phosphorus mean and S.E.	Calcium (blood) mean and S.E.	[Ca] [P] milli-mols per l.	Calcium (muscle) mean and S.E.
			mg. per 100 cc.	*mg. per 100 cc.*		*mg. per 100 g.*
14	1	8	4.3 ± 0.2	11.8 ± 0.7	4.6	—
15	2	10	4.3 ± 0.1	10.2 ± 0.6	3.5	28.1 ± 3.3
16	3	16	5.0 ± 0.2	12.9 ± 0.8	5.3	19.9 ± 2.9
24	11	10	4.9 ± 0.2	12.4 ± 0.3	4.9	19.4 ± 2.6
29	16	5	4.7 ± 0.2	12.1 ± 0.7	4.8	16.7 ± 3.2
41	28	11	5.2 ± 0.1	9.3 ± 0.3	3.9	19.4 ± 1.2
70	57	11	6.1 ± 0.2	12.4 ± 0.6	5.9	17.9 ± 1.5

TABLE XIX

Total, Diffusible, and Nondiffusible Calcium in Blood of Normal Guinea Pigs

		Mean ± standard error, mg. per 100 cc.			
Age *wks.*	No. of determinations	Total Ca (*a*)	Diffusible Ca (*b*)	Non-diffusible Ca (*a*)-(*b*)	Per cent diffusible Ca $\frac{(b)}{(a)} \times 100$
15	5	11.29 ± 1.40	7.49 ± 1.94	3.80	66.4
30	4	10.18 ± 0.00	6.23 ± 0.20	3.95	61.2
67	3	9.90 ± 0.09	4.25 ± 0.27	5.65	42.9
120	3	8.60 ± 0.86	4.08 ± 0.27	4.52	47.4
Average					54.5

TABLE XX

Total, Diffusible, and Nondiffusible Calcium in Blood of Guinea Pigs Deficient in Antistiffness Factor

			Mean ± standard error, mg. per 100 cc.			
Age *wks.*	Time on diet *wks.*	No. of determinations	Total Ca (*a*)	Diffusible Ca (*b*)	Non-diffusible Ca (*a*)-(*b*)	Per cent diffusible Ca $\frac{(b)}{(a)} \times 100$
17	4	9	10.81 ± 0.33	3.68 ± 0.25	7.13	34.1
19	6	11	12.15 ± 0.95	4.05 ± 0.43	8.10	33.9
25	12	3	10.51 ± 0.29	3.38 ± 0.27	7.14	32.1
52	39	8	11.18 ± 0.59	2.72 ± 0.68	8.46	24.4
73	60	6	11.13 ± 0.52	3.23 ± 0.04	7.90	29.0
Average						30.7

normal levels, after supplementation of the diet with the antistiffness factor was very slow (Table XXI).

c. Protein Metabolism. In cases of an abnormality in the calcium concentration in blood serum, the possibility exists that it is associated with an abnormal distribution of serum proteins. A derangement of the protein distribution was actually found in guinea pigs suffering from the deficiency of the antistiffness factor (van Wagtendonk and Zill, 1945). The albumin/globulin ratio in the blood plasma of these guinea pigs was considerably lower than that found in normal animals of the same age group (Tables XXII, XXIII). There was no speedy recovery from the abnormal protein distribution following the administration of the curative agent (Table XXIV). In dosages of 0.5 and 5 µg. (5 consecutive

TABLE XXI

Effect of Administration of Antistiffness Factor on Distribution of Total, Diffusible, and Nondiffusible Calcium in Blood of Guinea Pigs (39 Weeks on Deficient Diet)

No. of determinations	Dosage µg.	Mean ± standard error, mg. per 100 cc.			Per cent diffusible Ca $\frac{(b)}{(a)} \times 100$
		Total Ca (*a*)	Diffusible Ca (*b*)	Non-diffusible Ca (*a*)-(*b*)	
8		11.18 ± 0.59	2.72 ± 0.68	8.46	24.4
7	0.05[1]	11.43 ± 0.26	3.52 ± 0.38	7.91	31.0
5	0.5[2]	9.92 ± 0.63	3.58 ± 0.57	6.34	36.1
5	5[3]	10.51 ± 0.29	4.75 ± 0.31	5.76	45.1
5	50[4]	10.21 ± 0.09	5.62 ± 0.15	4.60	55.0

[1] 0.01 µg. per day during the last 5 days of the experiment.
[2] 0.1 µg. per day during the last 5 days of the experiment.
[3] µg. per day during the last 5 days of the experiment.
[4] 1 µg. per day during the last 50 days of the experiment.

TABLE XXII

Distribution of Nitrogen in Plasma of Guinea Pigs Receiving Stock Diet

Age *wks.*	No of determinations	Mean and standard error, mg. per 100 cc.					$\frac{\text{Albumin}}{\text{Globulin}}$
		Total N	Total protein N	Non-protein N	Albumin N	Globulin N	
10	5	719 ± 17	691 ± 16	28 ± 3	357 ± 36	334 ± 34	1.07
12	5	684 ± 13	663 ± 15	21 ± 3	308 ± 42	356 ± 52	0.87
16	15	749 ± 14	721 ± 16	28 ± 2	342 ± 20	380 ± 24	0.95
72	10	707 ± 52	639 ± 53	68 ± 4	319 ± 31	320 ± 32	1.00
72*	10	842 ± 69	759 ± 67	83 ± 5	410 ± 91	348 ± 37	1.18

* This group of animals had received a raw milk diet for 70 weeks.

TABLE XXIII

Distribution of Nitrogen in Plasma of Guinea Pigs Receiving Diet Deficient in Antistiffness Factor

Age *wks.*	Time on diet *wks.*	No. of determinations	Mean and standard error, mg. per 100 cc.					Albumin/Globulin
			Total N	Total protein N	Non-protein N	Albumin N	Globulin N	
14	1	15	749 ± 23	684 ± 27	56 ± 4	228 ± 27	466 ± 31	0.49
15	2	15	844 ± 16	781 ± 19	58 ± 3	345 ± 16	441 ± 26	0.65
16	3	15	795 ± 15	750 ± 17	45 ± 4	204 ± 20	546 ± 32	0.37
20	7	8	911 ± 26	827 ± 26	84 ± 13	215 ± 31	612 ± 38	0.35
41	28	15	923 ± 22	855 ± 23	67 ± 3	224 ± 42	631 ± 48	0.36
70	57	16	890 ± 26	816 ± 29	82 ± 3	298 ± 37	533 ± 45	0.56

TABLE XXIV

Effect of "Cure" with Antistiffness Factor on Distribution of Plasma Nitrogen of Guinea Pigs Raised on Skim Milk Diet

Experiment No.	Age	Time on diet	Deficient							Supplement of antistiffness factor							
			No. of determinations	Mean ± standard error, mg. per 100 cc.					Albumin/Globulin	Dosage of antistiffness factor	No. of determinations	Mean ± standard error, mg. per 100 cc.					Albumin/Globulin
				Total N	Total protein N	Non-protein N	Albumin N	Globulin N				Total N	Total protein N	Non-protein N	Albumin N	Globulin N	
	wks.	*wks.*								µg.							
1	20	7	8	911 ± 26	827 ± 26	84 ± 13	215 ± 31	612 ± 38	0.35	0.5[1]	7	910 ± 11	823 ± 47	86 ± 9	224 ± 49	599 ± 45	0.37
2	32	19	5	870 ± 18	779 ± 20	91 ± 8	213 ± 69	567 ± 74	0.37	0.5[1]	10	920 ± 22	841 ± 22	79 ± 1	296 ± 40	544 ± 31	0.54
	32	19								5[2]	5	901 ± 33	821 ± 34	80 ± 3	337 ± 35	480 ± 85	0.70
3	81	68	8	990 ± 20	884 ± 21	106 ± 10	204 ± 30	680 ± 45	0.30	50[3]	10	800 ± 26	718 ± 20	80 ± 2	360 ± 25	358 ± 20	1.00
	81	68								100[4]	8	840 ± 20	758 ± 20	82 ± 1	410 ± 30	341 ± 20	1.18
4	23	10								1[5]	14	887 ± 11	816 ± 11	71 ± 3	402 ± 21	413 ± 19	0.97

[1] Administered in daily dosages of 0.1 µg. during the last 5 days of the experiment.
[2] Administered in daily dosages of 1 µg. during the last 5 days of the experiment.
[3] Administered in daily dosages of 1 µg. during the last 50 days of the experiment.
[4] Administered in daily dosages of 1 µg. during the last 100 days of the experiment.
[5] Administered every other day during the experiment.

TABLE XXV

Distribution of Nucleotides and Nucleosides in Liver, Kidneys, and Blood of Normal and Deficient Guinea Pigs

Age *wks.*	Diet	Time on diet *wks.*	No. of determinations	Nucleotide N Mean ± s.e., mg. %			Nucleoside N Mean ± s.e., mg. %		
				Liver	Kidney	Blood	Liver	Kidney	Blood
15	Stock	15	15	18.4 ±1.0	15.6 ±0.5	5.1 ±0.3	12.6 ±1.0	4.7 ±0.8	4.4 ±0.6
70	Stock	70	10	19.9 ±0.6			12.1 ±0.9		
21	Deficient	6	15	14.2 ±0.7	14.4 ±1.0	4.0 ±0.4	6.7 ±0.4	4.0 ±0.2	3.7 ±0.3
31	Deficient	16	16	13.5 ±1.0	9.3 ±0.8	4.0 ±0.5	5.3 ±0.6	3.1 ±0.5	2.3 ±0.1
51	Deficient	36	14	10.2 ±0.8	10.3 ±0.9	4.0 ±0.3	4.9 ±0.4	3.3 ±0.3	1.2 ±0.2
84	Deficient	69	6	6.9 ±0.6	10.2 ±0.7	3.2 ±0.3	2.3 ±0.3	2.0 ±0.4	1.8 ±0.1

TABLE XXVI

Effect of Administration of Antistiffness Factor on Distribution of Nucleotides and Nucleosides in Liver, Kidneys, and Blood of Guinea Pigs on Skim Milk Diet

Age *wks.*	Time on diet *wks.*	Dosage of anti-stiffness factor µg.	No. of determinations	Nucleotide N Mean ± s.e., mg. %			Nucleoside N Mean ± mg. %		
				Liver	Kidney	Blood	Liver	Kidney	Blood
36	21	0	5	8.8 ±0.9	6.3 ±0.7	4.2 ±0.3	4.9 ±0.2	4.2 ±0.1	3.1 ±0.2
36	21	0.5[1]	7	18.6 ±0.5	8.7 ±0.5	4.1 ±0.1	7.9 ±0.4	3.8 ±0.2	3.5 ±0.2
36	21	5.0[2]	10	18.4 ±0.6	12.0 ±0.8	5.2 ±0.2	9.0 ±0.1	4.0 ±0.3	4.0 ±0.5
36	21	10.0[3]	8	19.0 ±0.9	19.9 ±0.6	5.4 ±0.1	11.6 ±0.6	4.9 ±0.1	4.7 ±0.4
51	36	1.0[4]	16	19.0 ±0.9	14.0 ±1.0	5.0 ±0.5	12.1 ±1.0	4.1 ±0.4	4.2 ±0.5

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[1] Administered in daily dosages of 0.1 µg. during the last 5 days of the experiment.

[2] Administered in daily dosages of 1 µg. during the last 5 days of the experiment.

[3] Administered in daily dosages of 1 µg. during the last 10 days of the experiment.

[4] Administered every other day during the whole course of the experiment.

dosages of 0.1 and 1 μg.) the influence is slight, although some improvement could be noted. If, however, the antistiffness factor was given for a longer period a return to normal conditions results. An abnormal distribution of the serum proteins could be prevented by a continuous administration of the factor.

TABLE XXVII

Uric Acid Content of Blood of Normal Guinea Pigs and of Guinea Pigs Deficient in Antistiffness Factor

Age	Normal		Deficient		
	No. of determinations	Mean ± S.E.	Time on diet	No. of determinations	Mean ± S.E.
wks.		*mg. per 100 cc.*	*wks.*		*mg. per 100 cc.*
15	4	5.81 ± 0.07			
21			6	15	5.89 ± 0.12
30	8	4.56 ± 0.09	16	16	4.30 ± 0.10
51			36	14	4.11 ± 0.13
70	10	5.20 ± 0.16	55	6	3.66 ± 0.09
84			69	6	4.31 ± 0.15
Average...........		5.19			4.45

TABLE XXVIII

Oxygen Consumption and Anaerobic Glycolysis of Liver Slices of Normal and Deficient Guinea Pigs

Diet	Q_{O_2} (20 determinations)	$Q^{N_2}_{CO_2}$	
	Mean ± S.E.	No. of determinations	Mean ± S.E
Stock	2.83 ± 0.18 (1.83–3.98)	18	3.04 ± 0.20 (1.95–4.10)
Deficient	4.08 ± 0.20 (3.08–5.07)	19	1.69 ± 0.17 (0.69–2.80)

The figures in parentheses indicate the range of observed values.

d. Purine Metabolism. The derangement of the acid-soluble P distribution involves a decrease of the labile nucleotide P and an increase of the stable nucleotide P. A further breakdown of the nucleotides occurs in the deficient animal (van Wagtendonk, 1947). The concentrations of the nucleotides and nucleosides decreased in the liver and kidneys and in the blood during the course of the deficiency (Tables XXV and XXVI). Administration of the antistiffness factor again resulted

TABLE XXIX

Oxygen Consumption and Anaerobic Glycosis of Kidney Cortex Slices and Muscle Strips of Normal and Deficient Guinea Pigs

Diet	Time on diet *wks.*	Kidney cortex						Muscle			
		Q_{O_2}		Total activity,[1] mean	$Q_{CO_2}^{N_2}$		Total activity,[1] mean	Q_{O_2}		$Q_{CO_2}^{N_2}$	
		No. of determinations	Mean ± S.E.		No. of determinations	Mean ± S.E.		No. of determinations	Mean ± S.E.	No. of determinations	Mean ± S.E.
Stock	13–90	12	10.90 ± 0.36 (8.00–14.70)[2]	3.38	9	4.94 ± 0.44 (2.52–7.38)	1.53	12	2.73 ± 0.15 (1.77–3.49)	9	4.70 ± 0.38 (2.43–5.94)
Skim milk	2– 3	5	10.40 ± 0.40 (8.80–11.70)	3.21				5	2.56 ± 0.12 (1.72–3.34)		
Skim milk	9–10	3	11.40 ± 0.53 (10.90–12.30)	3.52				3	2.52 ± 0.90 (2.31–2.70)		
Skim milk	13–14	3	8.30 ± 0.36 (7.60–9.10)	3.08				4	2.60 ± 0.09 (2.39–2.84)		
Skim milk	20–24	6			8	5.40 ± 0.52 (3.08–6.55)	2.00			8	4.73 ± 0.40 (3.39–6.58)
Skim milk	30–35	4	8.50 ± 0.33 (8.13–9.45)	3.12	3	5.40 ± 0.06 (5.23–5.47)	2.00	3	2.26 ± 0.05 (2.22–2.33)		
Skim milk	66–77	4	6.70 ± 0.55 (5.10–7.90)	3.00	3	5.11 ± 0.25 (4.48–5.58)	2.50	3	1.95 ± 0.25 (1.54–2.54)	3	4.25 ± 0.47 (3.63–4.78)

[1] Total activity is Q_{O_2} (or $Q_{CO_2}^{N_2}$) times per cent kidney in the guinea pig.

[2] The figures in parentheses indicate the range.

in a return to normal values. There was no significant difference in the uric acid content of normal and of deficient animals (Table XXVII).

e. Tissue Metabolism. An abnormal aerobic and anaerobic glycolysis of liver, kidney, and muscle tissues could be expected as a result of the changes in the phosphorus distribution in these tissues. That this was actually the case was demonstrated in experiments by van Wagtendonk and Simonsen (1946). The rate of oxygen consumption of liver slices increased while the anaerobic glycolysis rate decreased within one week to a low level which remained constant for the remainder of the period of investigation (Table XXVIII). The liver slices of animals that had received the deficient diet for 77 weeks had a $Q_{O_2} = 4.12$ and a $Q^{N_2}_{CO_2} = 1.95$.

A different picture was obtained when the respiratory rate and the anaerobic glycolysis of kidney cortex and muscle tissue were determined (Table XXIX). A decrease of the oxygen consumption of the kidney cortex became apparent only after the deficiency had progressed beyond the tenth week. The decrease then was very gradual. Apparently no significant deviations from the normal values for the $Q^{N_2}_{CO_2}$ were found. However, it was found that the kidneys of deficient guinea pigs were from 1.5 to 2 times larger than of those of normal animals of the same age, (see also Harris and Wulzen, 1949). Since this is due to tissue proliferation (there is no difference in the wet to dry ratio of these tissues, as reported in Table XXX) the value of these processes in the intact organ would be greater. It can be seen from Table XXIX that the Q_{O_2} (total activity) would remain constant; the $Q^{N_2}_{CO_2}$ (total activity) on the other

TABLE XXX

Water Content of Liver, Muscle, and Kidney Tissues of Guinea Pigs Raised on Stock Diet and on Diet Deficient in Antistiffness Factor

Tissue samples were taken immediately after death and exsanguination, and dried to constant weight at 105°. All determinations were made in duplicate. The Q_{O_2} and $Q^{N_2}_{CO_2}$ on a wet weight basis may be converted to Q_{O_2} and $Q^{N_2}_{CO_2}$ on a dry weight basis by multiplication by the wet to dry ratio.

Tissue	Diet	No. of determinations	Mean % water	Standard deviation	Standard error	Mean wet to dry ratio
Liver	Stock	17	73.50	0.23	0.055	3.77
	Skim milk	23	72.15	0.23	0.058	3.64
Muscle	Stock	16	79.20	0.49	0.123	4.80
	Skim milk	24	77.90	0.49	0.099	4.51
Kidney	Stock	15	78.80	0.34	0.088	4.70
	Skim milk	21	79.00	0.35	0.076	4.73

TABLE XXXI

Influence of Administration of Antistiffness Factor to Guinea Pigs Deficient in This Factor on Oxygen Consumption and Anaerobic Glycolysis of Liver, Kidney Cortex, and Muscle

Diet (35 wks.)	Liver				Kidney cortex				Muscle			
	Q_{O_2}		$Q_{CO_2}^{N_2}$		Q_{O_2}		$Q_{CO_2}^{N_2}$		Q_{O_2}		$Q_{CO_2}^{N_2}$	
	No. of determinations	Mean ± S.E.	No. of determinations	Mean ± S.E.	No. of determinations	Mean ± S.E.	No. of determinations	Mean ± S.E.	No. of determinations	Mean ± S.E.	No. of determinations	Mean ± S.E.
Deficient	3	4.70 ± 0.52 (3.78–5.92)	3	1.69 ± 0.110 (1.48–1.96)	4	8.50 ± 0.33 (8.13–9.45)	3	5.40 ± 0.06 (5.23–5.46)	3	2.26 ± 0.05 (2.19–2.31)	3	5.00 ± 0.03 (4.21–5.38)
Deficient and antistiffness factor	6	2.78 ± 0.15 (2.33–3.36)	6	2.18 ± 0.39 (1.00–4.10)	7	7.63 ± 0.42 (6.45–9.50)	7	6.16 ± 0.44 (4.61–8.36)	6	2.19 ± 0.12 (1.82–2.56)	6	4.88 ± 0.55 (2.88–6.42)

The figures in parentheses indicate the range.

hand would increase. Some objections could be raised against this extrapolation, since we only determined the rates of respiration and of anaerobic glycolysis in the kidney cortex, while the proliferation of the kidney is not solely due to proliferation of this tissue. However, the cortex is certainly the most active metabolizing part of the kidney and, since this tissue, too, has proliferated, a calculation on the above basis would seem justified.

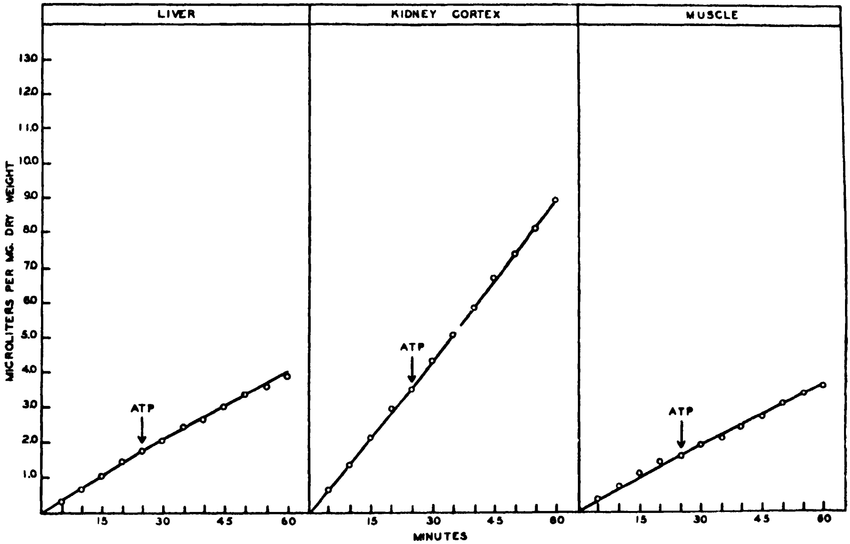


FIG. 24. The oxygen consumption of tissues from deficient guinea pigs (36 weeks deficient) before and after addition of adenosinetriphosphate (ATP). The side cup contained ATP dissolved in phosphate-Ringer's buffer according to the procedure of Dickens and Greville (0.5 cc., 0.012*M*) adjusted to a pH of 7.4. The main compartment contained 1.5 cc. of phosphate-Ringer's solution. After the contents of the two cups (indicated by the arrows) were mixed, the final concentration of ATP was 0.003*M*. The points on the curves represent the averages from four different animals (triplicate determinations per tissue).

The respiratory rate of muscle tissue decreased significantly only after the 13th to 14th week, coinciding with the decrease in concentration of creatine phosphate and the adenosinephosphates. A slight, but significant decrease in the anaerobic glycolysis was found only after the animals had received the deficient diet for a period of over one year.

Administration of the antistiffness factor in five daily dosages of 2 μg. each, to animals that had received the deficient diet for 35 weeks, had a definite effect on the respiratory rate and the anaerobic glycolysis of the liver (Table XXXI). A distinct tendency to a return to normal values could be observed, although the normal rates were not completely obtained. No significant change could be found in the respiratory rate of the kidney cortex, although a small decrease was noticeable. The rate of anaerobic glycolysis showed an increase rather than a decrease. The two metabolic processes in the muscle were not effected by this short term administration of the antistiffness factor.

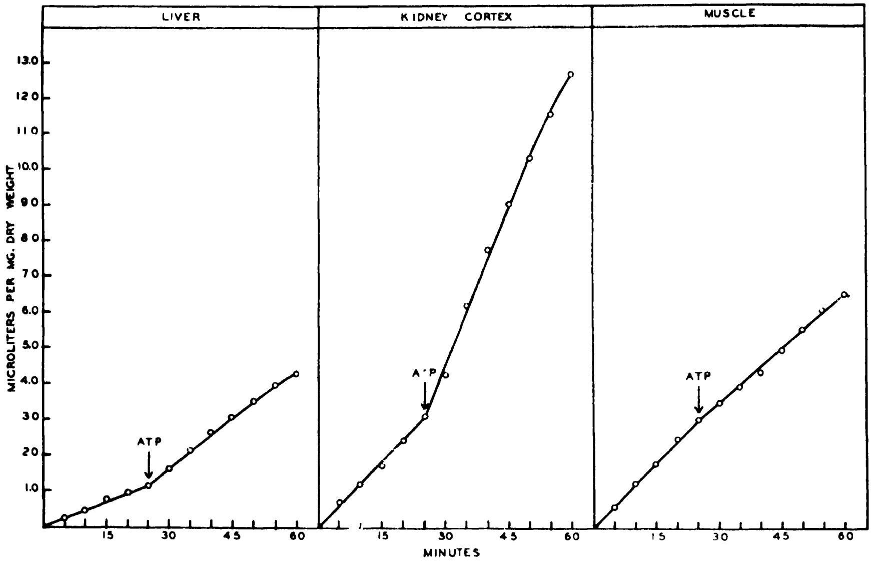


FIG. 25. Anaerobic glycolysis of tissues from deficient guinea pigs (36 weeks deficient) before and after addition of adenosinetriphosphate (ATP). The side cup contained ATP dissolved in bicarbonate-Ringer's solution, according to the method of Krebs and Henseleit (0.5 cc., 0.018*M*), saturated with CO_2 and carefully adjusted to pH 7.4. A careful adjustment of the pH of the solutions in the two compartments is essential. If the pH of the ATP solution is 0.1 unit lower than that of the substrate, mixing will cause a change of pressure corresponding to 20 mm. of Brodie's fluid, due to liberation of CO_2. The main compartment contained 2.5 cc. of bicarbonate-Ringer's solution treated similarly. After the contents of the two cups were mixed (indicated by the arrows), the final concentration of ATP was 0.003*M*. The points on the curves represent the averages from six animals (triplicate determinations per tissue).

These results are an indication that the antistiffness factor exerts its regulatory effect on tissue metabolism indirectly through its influence upon the functioning of the adenylic acid system. If this were really the case, addition of adenosinetriphosphate (ATP) to the substrate should restore both aerobic and anaerobic reactions to the rate found in normal tissues. Addition of ATP to aerobically metabolizing liver, kidney cortex and muscle tissue caused a slight increase in Q_{O_2} only in kidney cortex tissue slices (Fig. 24). However, addition of ATP had a striking effect on the anaerobic process in all three tissues. Large increases were found in liver and kidney cortex tissue upon the addition of ATP. A slight decrease in the rate of this process was found upon addition of ATP to metabolizing muscle tissue (Fig. 25).

The high respiratory rate of the deficient liver might indicate that glycogen is oxidized directly instead of being degraded via the normally occurring phosphorylating oxidation-reduction cycles. A low concentration of glycogen in the liver would then result. This was actually the case (Table XXXII). Administration of 2 μg. of the antistiffness factor per day resulted in a gradual return to normal values.

TABLE XXXII

Effect of Varying Dosages of Antistiffness Factor on Liver Glycogen of Deficient Guinea Pigs

Diet	Time on diet *wks.*	No. of determinations	Days of treatment, 2 μg. per day	Glycogen, wet weight %
Deficient	3–72	16		0.55 ± 0.06 (0.20–0.93)*
Deficient on cure	10	2	1	0.08
				0.22
Deficient on cure	10	2	2	0.11
				0.24
Deficient on cure	10	2	3	0.15
				0.18
Deficient on cure	10	3	4	0.93
				2.38
				5.94
Deficient on cure	10	2	5	2.88
				4.70
Deficient on cure	10	3	6	1.98
				2.91
				3.82
Stock		10		2.98 ± 0.29 (1.51–4.36)*

* Means ± S.E. (values in parentheses indicate the range).

The *in vitro* addition to liver homogenates of deficient guinea pigs of the purified antistiffness factor (see Section III, 4) in a concentration of 3×10^{-7} *M* caused a marked and significant increase in the $Q^{N_2}_{CO_2}$ of this homogenate (Simonsen and van Wagtendonk, 1947) (Table XXXIII). The addition of cholesterol, and of a synthetic unbranched aliphatic alcohol ($C_{29}H_{59}OH$) were without effect. Ergosterol and a natural product isolated from cane juice (m.p. 82.5–84°), identified as a C_{28} or

TABLE XXXIII

Anaerobic Glycolysis of Normal and Deficient Liver Homogenated with and without in Vitro Addition of Antistiffness Factor and Other Substances

Diet	No. of determinations[1]	Compound tested	$Q_{CO_2}^{N_2}$ (Mean ± standard error)
Deficient	6		0.43 ± 0.04 (0.26–0.61)[2]
		Antistiffness factor, 164°	0.59 ± 0.05 (0.38–0.76)
Deficient	5		0.39 ± 0.03 (0.27–0.47)
		Natural C_{29}	0.42 ± 0.02 (0.41–0.45)
Deficient	4		0.44 ± 0.01 (0.33–0.55)
		Synthetic C_{29}	0.43 ± 0.01 (0.33–0.52)
Deficient	5		0.39 ± 0.07 (0.26–0.46)
		Ergosterol	0.56 ± 0.06 (0.51–0.68)
Deficient	4		0.41 ± 0.02 (0.37–0.45)
		Cholesterol	0.41 ± 0.01 (0.39–0.45)
Stock	6		1.06 ± 0.17 (0.53–1.52)
		Antistiffness factor, 164°	1.04 ± 0.17 (0.52–1.53)

[1] Each determination represents the average of six flasks.

[2] The values in parentheses indicate the range.

TABLE XXXIV

Comparison of Rates of Anaerobic Glycolysis of Liver Homogenates from Deficient Guinea Pigs, with and without in Vitro Addition of Antistiffness Factor (164° Melting Point)

The results represent $Q_{CO_2}^{N_2}$.

Time on diet *wks.*	Without added factor	With added factor	Increase	Per cent increase
6	0.45	0.51	0.06	13.3
6	0.55	0.76	0.21	38.2
6	0.43	0.58	0.15	34.9
6	0.61	0.75	0.14	23.0
10	0.43	0.56	0.13	30.2
20	0.26	0.38	0.12	46.2
Mean values............	0.455	0.59	0.135	31.0

C_{29} unbranched aliphatic alcohol and probably still containing traces of the active high-melting compound also showed activity. The average per cent increase found with the antistiffness factor added was 31% (Table XXXIV).

3. Conclusions

The injury caused by the administration of the skim milk diet differs from those previously reported for other deficiencies because of the character and distribution of the lesions. Some of the lesions resemble those found during ascorbic acid deficiency, others resemble those found during vitamin E deficiency. However, the lesions found during the deficiency of the antistiffness factor occur when the diet is more than adequately supplied with these vitamins. Moreover, some of the typical aspects of the aforementioned deficiencies were absent, as for example the pigmentation occurring in the uterine and other smooth muscles and in skeletal muscles of vitamin E deficient rats (Martin and Moore, 1936; Barrie, 1938; Hessler, 1941; Demole, 1942; Mason and Emmel, 1945). The deficiency described by Woolley and coworkers (Woolley, 1942; Woolley and Sprince, 1944, 1945; Sober *et al.*, 1942) is not identical with the deficiency of the antistiffness factor.

The antistiffness factor plays a definite role in the metabolism of the guinea pig, and can therefore be classified as a vitamin or essential nutrilite. What then would be the role of this vitamin in metabolism?

It is now generally recognized that vitamins are constituents or modifiers of enzyme systems (Warburg *et al.*, 1935; Green, 1942). Hickmann and Harris (1946) have listed the activities in which a vitamin may engage:

1. Primary—the vitamin cooperates.
    - *a.* The vitamin is an enzyme.
    - *b.* The vitamin is part of an enzyme molecule.
    - *c.* The vitamin cooperates within an enzymic reaction.
2. Secondary—the vitamin modifies or is modified.
    - *a.* The vitamin preserves another vitamin or enzymic agent from destruction-sparing action or sparing agent.
    - *b.* The vitamin accelerates destruction-spending action or spending agent.
    - *c.* The vitamin replaces or is replaced by another vitamin or antivitamin or replacing agent.
    - *d.* The requirements for the vitamin are altered by nutritional factors, not themselves vitamins.

According to this classification, the antistiffness factor would belong to class 1*b* for the reasons listed below.

The studies on tissue metabolism indicate that this factor plays an important role in the energy metabolism through its influence on the adenylic acid system. It might either restrain the activity of the adenosinetriphosphatase and the adenosinepyrophosphatase, or act as a prosthetic group which promotes the rephosphorylation of adenylic acid. In the first case a deficiency of the factor would promote the rapid breakdown of any adenosinetriphosphate formed during the metabolic cycles, resulting in an increase of inorganic phosphate and adenylic acid (or further breakdown products) in the tissues. The metabolic cycles could not be slowed down, but would proceed at a higher rate, since adenylic acid would become available in large amounts to act as an acceptor for the energy-rich phosphates formed during both the anaerobic glycolysis and the following tricarboxylic acid and cytochrome cycles. The rapid dephosphorylation of the formed adenosinetriphosphate would, however, seriously interfere with the energy metabolism, because the conversion of energy-rich phosphate compounds into inorganic phosphorus would mean a loss of the stored energy which would then not be available for other reactions requiring the expenditure of energy.

If, however, the antistiffness factor would act as a prosthetic group for the enzyme responsible for the phosphorylation of adenylic acid, a more direct interference with the normal metabolic cycles would result. The phosphorylation of adenylic acid would then be blocked in the deficient tissue, and the adenylic acid system would not be able to act as a phosphate acceptor for the energy-rich phosphate groups formed during the metabolic cycles. Especially the anaerobic glycolysis would then be slowed down.

The results obtained in the various investigations indicate that the latter viewpoint, e.g., the antistiffness factor acts as a part of an enzyme system, is correct. In the deficient animal there is a definite shift towards the oxidative processes. The anaerobic glycolysis is decreased considerably, indicating that the normal pathway of carbohydrate utilization leading towards the hub of pyruvic acid is being by-passed. Warburg and coworkers (1935, 1937) have shown that animal tissues can oxidize glucose-6-phosphate directly to phosphogluconic acid, and there is increasing evidence from other laboratories that a series of reactions, possibly starting with the direct oxidation of glucose-6-phosphate intervenes early in the glycolysis of carbohydrate (Lundsgaard, 1930a, b, c; Barker *et al.*, 1939).

Since a direct oxidation of glucose to phosphogluconic acid will of necessity provide less energy to the animal organism than the normal pathway of carbohydrate utilization, a by-passing of the fermentation reactions will lead to a more extensive use of the glycogen formed in the liver. This then would lead to a decrease in the concentration of liver glycogen. It was actually found that the amount of glycogen in the liver of deficient animals is decreased to about 1–5% of that present in normal animals.

The addition of adenosinetriphosphate results in a return to normal conditions of the aerobic and anaerobic metabolic processes. The increase in the respiratory rate and the decrease in the anaerobic glycolysis in deficient liver slices are reversible by the *in vivo* administration of the antistiffness factor for 5 days. *In vitro* addition of adenosinetriphosphate and *in vitro* addition of the antistiffness factor in a concentration of $3 \times 10^{-7}M$ tend to restore the glycolysis rate of deficient tissue slices and minces respectively. It seems therefore highly probable that the antistiffness factor cooperates within the adenylic acid enzyme system.

That not all the observed changes are as freely reversible should not be surprising. Some of the aberrations constitute a severe gross change in the tissues, which would require time to be restored to the normal state. Furthermore many of these changes might be considered to be secondary changes due to the primary derangement of the energy metabolism.

A few words need to be said about the excessive calcification found during the deficiency. It has been pointed out by others (Hogan and Regan, 1946; Petering *et al.*, 1948; Anon., 1948) that the disarranged calcium and phosphorus metabolism might be due to an imbalance of these minerals in the milk diet. The nature of the calcium deposits (Hogan and Regan, 1946) obtained when guinea pigs were raised on a purified diet containing adequate calcium and 0.6 g. of phosphorus per 100 g. ration was quite similar to that seen in the guinea pigs when raised on the deficient milk diet. The P content of the diet of Hogan and Regan is 0.9%, which compares with 1.0% for the skim milk diet. That a high P content of the diet has some influence on the P distribution was demonstrated by the fact that animals raised on a raw milk diet had a significantly higher inorganic P content of liver and kidneys. This does not, however, invalidate the proposed action of the antistiffness factor in the phosphorus metabolism. The deposition of calcium phosphate is not solely due to the imbalance of calcium and phosphorus. Since the albumin fraction is considerably lower in the deficient animal, a large proportion of the protein-bound calcium must have been liberated. This, coupled with the higher concentration of inorganic phosphate, will favor the formation of a colloidal calcium phosphate complex. This complex will precipitate in those tissues in which conditions are favorable and thus account for the large calcium phosphate deposits found in so many tissues. The deposition of calcium phosphate in the tissues is therefore neither a primary result of an imbalance of calcium and phosphorus in the diet, nor a primary result of the deficiency. It is dependent on two factors, i.e., the concentration of inorganic phosphate in the tissues and blood, and the concentration of albumin in the blood. The increased concentration of inorganic phosphate in the blood is again due to two other factors (1) the influence of the antistiffness factor on the adenylicacid system, and (2) the high phosphorus content of the diet.

The fact that these calcium deposits can be made to disappear by the administration of the antistiffness factor while the animals remain on the skim milk diet proves that the deposits are not solely due to the imbalance of calcium and phosphorus in the diet, but are certainly influenced by the absence of the antistiffness factor from the diet. Furthermore, no calcium deposits are ever found when guinea pigs are raised on a raw milk diet.

There is some indication that the antistiffness factor may also play a role in bacterial metabolism. Williams *et al.* (1949) suggested that the antistiffness factor might have a functional relationship to an, as yet, unidentified growth factor for *Lactobacillus bulgaricus*. This factor would then be of more universal importance than was originally thought.

IV. ISOLATION AND IDENTIFICATION OF THE ANTISTIFFNESS FACTOR

1. The Test for Activity

Animals on the deficient diet, which had developed wriststiffness were used for assays. The method consisted of a manual determination of wriststiffness in the following way: The foreleg of the guinea pig on the opposite side of the experimenter was extended posteriorly, close to the body wall of the animal, by pressing the thumb on the olecranon process and at the same time supporting the proximal and distal portions of the leg with the fingers. The leg should be as straight as possible. The disengaged hand of the operator was then used to superextend the foot gently by pressing upward on its medial aspect. The foot of a normal animal bends easily until it forms a right angle with the leg. The nutritional deficient animals were very sensitive towards the treatment and manifested pain at once when the foot was forced beyond the point of easy bending. This stiffness disappeared if active fractions were administered to the animals. The results are recorded in terms of a series of arbitrary units. A normal bending wrist is designated as 4, a complete rigid wrist as 1. Intermediate conditions are indicated by such symbols as 1.5, 2, 3, 3+, and 4−. 4^P indicates that, although normal mobility has been regained, the joint is still painful under manipulation. Both legs are used for the test. It has been our experience, gained through the handling of thousands of animals, that the most reproducible results are obtained when the stiffness reading does not decrease below the value of 3. Wrists stiffer than 3 do not respond so fast and as consistently as do wrists with a stiffness of 3. The response is always an all-or-none effect, and a gradation of cures, following administration of active fractions in graded amounts cannot be obtained. For example, an active fraction may give a complete cure in dosages of 100, 50, and 10 μg., but no effect can be noted when 5 μg. are administered. The cure with 10 μg. is just as fast as the cure with 100 μg. It has always been our policy to assay the fractions at the level of minimum curative dosage and assigning this amount the unit dosage.

It happens often that an appreciable number of animals in new shipments have wriststiffness when received. Apparently these animals were on a diet deficient in the antistiffness factor. Such animals should never be used for assay purposes. These observations have been corroborated by Petering *et al.* (1948). Sickness in the colony might also seriously effect the reproducibility of the assays. Whenever the above precautions are taken into account the assay will yield satisfactory results, with the limitations set forth above. Claims to the contrary (Christensen *et al.*, 1948) may be due to the fact that the optimal conditions for assay have not been fulfilled.

Petering and coworkers (1948) have improved the assay method by adding a control period. Animals that have been cured by supplementing the diet with active material, are subsequently placed back on the deficient diet, to determine whether relapse to a deficient state will ensue. Likewise, animals that have not responded to a supplement within the test period are given an effective supplement to be sure they were capable of reacting to a known curative agent. According to these authors, this is necessary, because of the fact that, as the animals become older and have gone through several test periods followed by curative periods, an appreciable number seem to become refractory to a known curative treatment.

We feel that the stiffness test is reliable especially if the recommendations by Petering and coworkers are observed. Although a more objective test would be desirable, the method has been a valuable tool in following the activity of various fractions during the course of the isolation.

2. Distribution of the Antistiffness Factor

The presence of antistiffness factor in powdered, dehydrated vegetables was determined in the following manner. Groups of three deficient guinea pigs with medium wriststiffness were given 0.5 g. dehydrated vegetable daily for 5 days. Most of the experiments were repeated 2 or 3 times and the results were averaged. The alleviation of wriststiffness was determined and expressed on an arbitrary mathematical scale (see Table XXXV). The vegetables varied from a very high curative power for endive to a very low power for kelp. Generally speaking, this order represents differences in the amounts of antistiffness factor in the vegetable powders used, but the mathematical values are in no sense absolute. (Unpublished data by R. Wulzen and M. E. Pruden.)

TABLE XXXV

*Experiments on Removal of Stiffness in Deficient Animals Through Use of Powdered, Dehydrated Vegetables**

Units of cure per wrist per day			
Endive	24.0	Dandelion	11.0
Carrot leaves	19.3	Beet root	10.6
Mustard greens	15.4	Carrot root	10.5
Kale	14.4	Red cabbage	10.2
Beet leaves	12.7	Cauliflower	9.2
Turnip leaves	12.3	Red radish	9.2
Celery leaves	12.0	Celery root	8.4
Jerusalem artichoke	11.6	Lemon	8.4
Green cabbage	7.3	Orange	5.8
Peas	6.8	Rhubarb	5.7
Asparagus	6.8	Black radish	5.4
Tomato	6.8	Grass	4.8
Watercress	6.4	Spinach	3.6
Lettuce	6.4	Dulse	2.8
Green beans	6.2	Alfalfa	2.4
Cucumber	6.2	Dill	2.0
Parsley	5.9	Kelp	0.4

* Vegetable powders (dehydrated at low temperature) furnished through the kindness of "California Vegetable Concentrates."

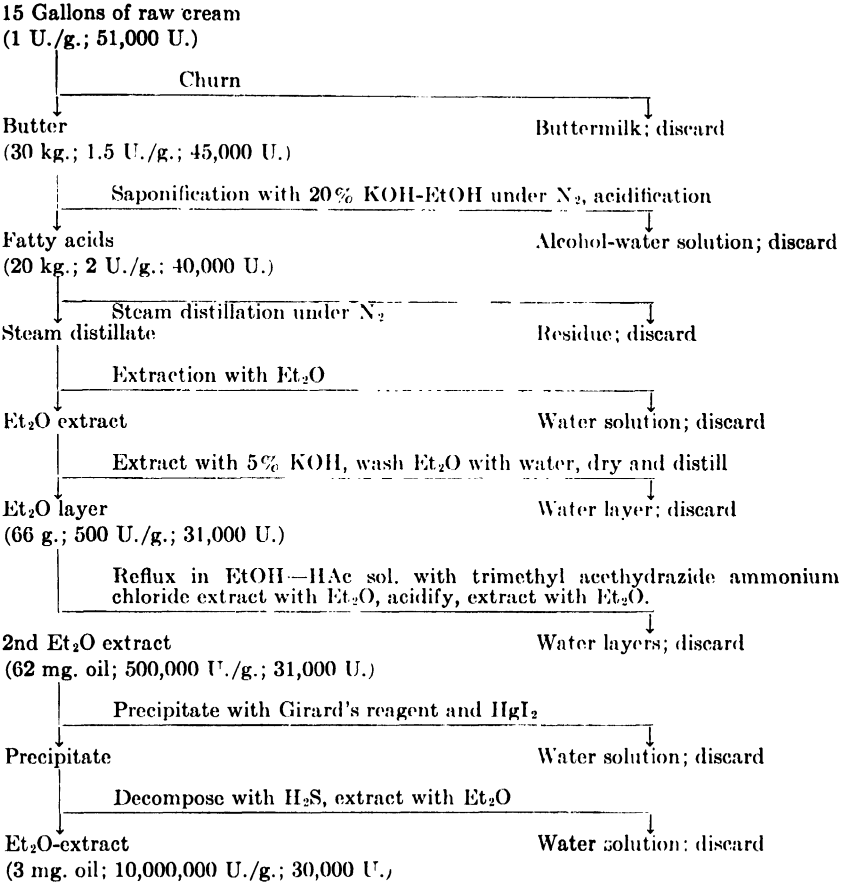
3. *Isolation from Raw Cream*

Attempts were made to isolate the active component from raw cream. The extraction procedure which was developed is given in Chart I.

Only 3 mg. of an oily substance, probably a ketone, could be isolated from 15 gallons of raw cream. The compound gave a precipitate with Girard's reagent no other typical ketone derivatives could be prepared. While there was not enough material available for a complete analysis, it could be established without any doubt that methyl vinyl ketone was not the active compound in raw cream (Gouley, 1941). The melting point of the mercuric iodide complex of the trimethyl acethydrazide

CHART I

EXTRACTION FROM RAW CREAM


15 Gallons of raw cream
(1 U./g.; 51,000 U.)
Churn
Butter
(30 kg.; 1.5 U./g.; 45,000 U.)
Buttermilk; discard
Saponification with 20% KOH-EtOH under N₂, acidification
Fatty acids
(20 kg.; 2 U./g.; 40,000 U.)
Alcohol-water solution; discard
Steam distillation under N₂
Steam distillate
Residue; discard
Extraction with Et₂O
Et₂O extract
Water solution; discard
Extract with 5% KOH, wash Et₂O with water, dry and distill
Et₂O layer
(66 g.; 500 U./g.; 31,000 U.)
Water layer; discard
Reflux in EtOH—HAc sol. with trimethyl acethydrazide ammonium chloride extract with Et₂O, acidify, extract with Et₂O.
2nd Et₂O extract
(62 mg. oil; 500,000 U./g.; 31,000 U.)
Water layers; discard
Precipitate with Girard's reagent and HgI₂
Precipitate
Water solution; discard
Decompose with H₂S, extract with Et₂O
Et₂O-extract
(3 mg. oil; 10,000,000 U./g.; 30,000 U.)
Water solution; discard

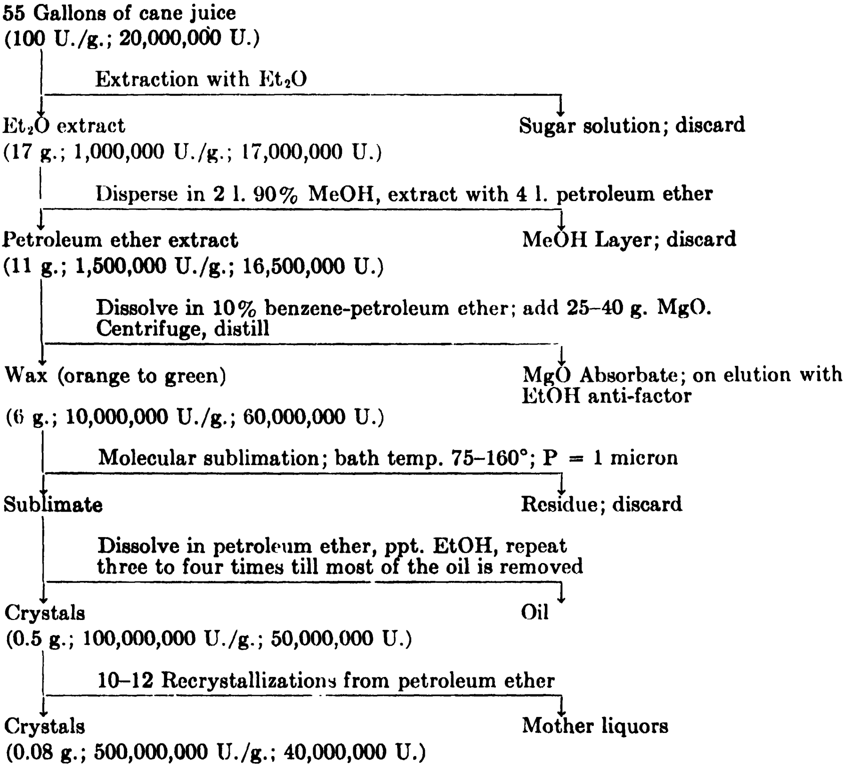
ammonium hydrochloride of the compound isolated from raw cream melted from 60–61°, while the mercuric iodide complex of methyl vinyl ketone melted from 120–121°.

4. Isolation from Crude Cane Juice

While the above experiments were in progress, it was noted that the stiffness syndrome did not develop in a group of animals that had received the skim milk diet for more than 5 months. Several possible sources of error were investigated without success until it was found that the skim milk had been prepared from milk of a herd that had received a liberal byfeeding of cane molasses. When a sample of this cane molasses

CHART II

EXTRACTION FROM CANE JUICE



55 Gallons of cane juice
(100 U./g.; 20,000,000 U.)

Extraction with Et_2O

Et_2O extract
(17 g.; 1,000,000 U./g.; 17,000,000 U.)

Sugar solution; discard

Disperse in 2 l. 90% MeOH, extract with 4 l. petroleum ether

Petroleum ether extract
(11 g.; 1,500,000 U./g.; 16,500,000 U.)

MeOH Layer; discard

Dissolve in 10% benzene-petroleum ether; add 25–40 g. MgO.
Centrifuge, distill

Wax (orange to green)
(6 g.; 10,000,000 U./g.; 60,000,000 U.)

MgO Absorbate; on elution with EtOH anti-factor

Molecular sublimation; bath temp. 75–160°; P = 1 micron

Sublimate

Residue; discard

Dissolve in petroleum ether, ppt. EtOH, repeat three to four times till most of the oil is removed

Crystals
(0.5 g.; 100,000,000 U./g.; 50,000,000 U.)

Oil

10–12 Recrystallizations from petroleum ether

Crystals
(0.08 g.; 500,000,000 U./g.; 40,000,000 U.)

Mother liquors

was tested, it was found that it was ten times more active than the raw cream. Subsequent tests showed that unheated cane juice was about a thousand times as active as raw cream. An isolation procedure for the extraction of the active component from molasses was worked out, which was later adapted to the extraction of raw cane juice. In total,

TABLE XXXVI

Stiffness Test

Dosage µg.	Course of cure													
	1st day		2nd day		3rd day		4th day		5th day		6th day		7th day	
10	2.5	2.5	3	3	4 −	4	4	4						
0.01	2.5	2.5	3.5	3 −	4 −	3+	4	3+	4	4 −	4	4P	4	4
0.002	3	2.5	3+	2.5	3.5	3.5	3.5	3.5	3.5	3.5	4 −	4 −	4	4
Control	2	2.5	2	2.5	2	2.5	2	2.5	2	2.5	2	2.5	2	2.5

6 tons of molasses and 7 tons of cane juice were extracted according to the procedure given in Chart II (van Wagtendonk and Wulzen, 1946).

The purified material melted from 81.5–82° and was active in a dosage of 0.002 μg. (Table XXXVI).

5. *Evidence for the Steroid Nature of the Antistiffness Factor*

The sharp melting point led us to believe that the isolated compound was homogeneous. However, carbon and hydrogen analyses of different batches of purified material failed to yield consistent values. Several purification procedures were tried without success. Repeated recrystallization from alcohol, followed by recrystallizations from acetone and cyclohexane yielded a compound, melting at 164–166° that was homogeneous according to the carbon and hydrogen analyses and which was active in dosages of at least 0.01 (Table XXXVII Ross *et al.*, 1949). The largest part of the originally isolated complex, which remained after removal of the active high-melting compound, melted from 84–85° and was inactive in the assay. This fraction was tentatively identified as an

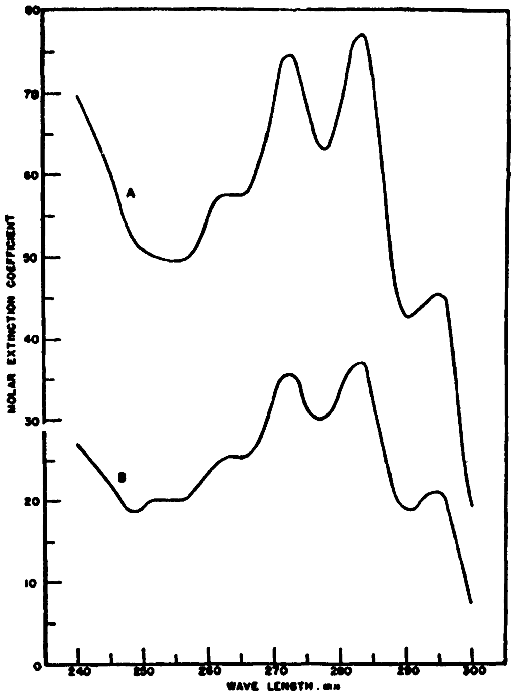


FIG. 26. Ultraviolet adsorption of the high melting compound (curve A) and its acetate (curve B).

unbranched aliphatic alcohol with the probable constitution of $C_{28}H_{57}OH$ or $C_{29}H_{59}OH$ (unpublished data by Ross, and van Wagtendonk).

The high melting compound was partially analyzed, but a complete elucidation of its structure was not accomplished, due to the small amount available (Ross *et al.*, 1949). The compound was tentatively identified as a steroid, with the probable formula of $C_{28}H_{46}O$. It gave a precipitate with digitonin, from which it could be recovered. The presence of one alcoholic group and of two double bonds was established. The ultraviolet adsorption spectra of the compound and its acetate are given in Fig. 26.

TABLE XXXVII

Therapeutic Test of High Melting Compound Carried Out on Guinea Pigs on Skim Milk Ration

Level of assay in μg. (5 times)	No. of animals	Cured	No cure
1	3	3	0
0.01	27	23	4

6. Conclusions

From the accumulated evidence it is clear that a compound of steroid nature, capable of relieving an induced stiffness in guinea pigs is present in cane juice. The identity of this compound with the one present in raw cream could not be established. Although the cream factor could be precipitated with Girard's ketone reagent, the possibility that the active factor coprecipitated with the bulky precipitate cannot be excluded. We never were able to obtain a precipitate with any other ketone reagent. The identity of the antistiffness factor with methyl vinyl ketone can be excluded on several grounds. The curative dose of methyl vinyl ketone is many times larger than of either the cream factor or the cane juice factor. Furthermore, methyl vinyl ketone has, in the curative dosage, extreme toxic effects.

A few words need to be said about the apparent discrepancies between the activity of the originally isolated substance and the purified high melting compound. This compound should have had a higher activity than the one isolated previously (Anon. 1948). Other workers have not been able to confirm the high potency of the originally isolated factor (Oleson *et al.*, 1947; Caldwell, 1948; Dasler and Bauer, 1949). The latter can be explained by the fact that probably not all the necessary precautions had been observed (see Sections IV, 1).

The former can only be explained by the fact that the antistiffness factor is an unstable compound, and deteriorates slowly, even in the purified state. Although precautions had been taken to keep inactivation at a minimum (low temperature, nitrogen atmosphere where possible) inactivation in the many steps involved in the purification procedure was practically unavoidable. Evidence for such a deterioration can be found in the following table (Table XXXVIII).

TABLE XXXVIII

Inactivation of the Antistiffness Factor with Time

Crude cane wax (step 3) was stored in a stoppered bottle in the refrigerator

Date tested	Amount tested	No. of animals	Cured	No cure
10-4-46	1 μg.	3	3	0
	1 μg.	3	1	2
10-19-46	1 μg.	3	0	3
11-16-46	10 μg.	3	0	3
1-11-47	1 mg.	9	1	8
4-10-47	4 mg.	5	2	3
6-12-47	6 mg.	3	1	2
12-29-47	6 mg.	3	0	3
2-3-48	5 mg.	3	0	3

V. Concluding Remarks

The syndrome caused by the deficiency of the antistiffness factor is characterized by the development of a stiffness at the wrist joint, and of a peculiar type of arteriosclerosis, necrosis, and calcification of the skeletal muscle and myocardium, deposition of calcium salts in the smooth muscle of the gastrointestinal tract, in kidneys and liver, and development of abscesses adjacent to bones and joints that frequently become calcified. The muscle injuries resemble changes described by others, but in view of the character and distribution of the other lesions, it is apparent that the etiology of the injury resulting from the administration of the skim milk diet differs from those previously reported. It seems improbable that the vitamin E intake was inadequate in view of the absence of pigmentation of the intestines and of the different picture of the creatine creatinine ratio during the course of the deficiency.

The various experiments, designed to establish the mode of action of the antistiffness factor in metabolism indicate that this factor acts as an integral part of an enzyme system, which is primarily involved in the energy metabolism. It is of great interest and importance that a compound of steroid nature is involved in the energy transfer system.

An upset of this system causes a chain of derangements in the various metabolic cycles, and ultimately gives rise to the many pathological aberrations found in the deficient animal.

The possibility exists that the antistiffness factor is a precursor of a compound normally synthesized in the animal body. Both, precursor and final compound would have a similar effect on the enzymic reactions.

A multitude of questions have remained unanswered, such as the structure of the antistiffness compound, its relation to the vitamin D complex, its possible relation to the hormones of the adrenal cortex, and its exact mode of action. It is to be hoped that future work on this important compound will give an answer to these questions.

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Vitamins and Metabolism in Neurospora

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I. INTRODUCTION

The general subject of vitamins and metabolism in the mold Neurospora has been considered in a number of previous reviews (Tatum, 1944; Beadle, 1945a and b, 1946, 1948; Horowitz *et al.*, 1945; Bonner, 1946, 1949). Although none of these reviews has been confined solely to a consideration of vitamins and metabolism in the mold a good many of the developments in this field have been discussed. As a consequence the present summary will provide details, for the most part, only on recent or current investigations.

Since one of the principal aims of the work with Neurospora has been to gain a better understanding of the biochemical relations of genes, enzymes, and biochemical reactions, it is surprising that a more organized and vigorous attack has not been made on the vitamin, coenzyme and enzymatic aspects of the problem. Not one of the known coenzymes has been shown with certainty to exist in Neurospora and a function of a coenzyme has been established in only one case (Umbreit *et al.*, 1946). A moderate amount of work has been reported on enzymes of Neurospora but not the quantity that would be expected from a consideration of the fundamental problem stated above. One reason for this deficiency is the fact that most of the mutants studied deal with biochemical reactions that have not been the subjects of previous investigations in enzymology. There is no evident reason why this difficulty cannot be surmounted. In any case it is clear that studies on metabolism, and metabolic patterns are so intimately concerned with genetic constitution that problems in both fields must be considered simultaneously. Certainly the vitamins, or their conjugates, participating as essential cellular catalysts, play most significant roles in linking genetic potentialities to the biochemical reactions that occur.

II. Mutants

Wild type strains of Neurospora can be grown satisfactorily on a synthetic medium consisting of a solution of inorganic salts, sugar and one vitamin, biotin, in distilled water (Beadle and Tatum, 1945; Westergaard and Mitchell, 1947). The remaining vitamins and other essential metabolites can be synthesized from these raw materials by the mold during growth. A mutation in the wild type strains often results in the loss of a step in the synthesis of a metabolite and thus causes a new nutritional requirement. These new strains have been most useful in studying mechanisms of metabolism, in spite of the fact that a mutation often produces profound physiological changes in addition to the introduction of the new growth requirement.

1. *Production of Mutants*

Mutants with requirements for vitamins and many other metabolites have been produced in Neurospora by treatment of the mold, at various stages of the life cycle with ultraviolet, X- and other types of radiation (Beadle and Tatum, 1945), as well as by treatments with the sulfur mustards (Horowitz *et al.*, 1946), and nitrogen mustards (Tatum, 1946; McElroy *et al.*, 1947). More recent evidence indicates that similar mutations can be obtained by use of other chemicals such as diazomethane (Westergaard, 1949) and organic peroxides (Dickey *et al.*, 1949). So far no clear evidence has been presented to show that one kind of treatment favors production of a particular kind of mutant although some genes apparently do undergo mutation more frequently than others.

McElroy *et al.* (1947) have provided evidence suggesting that young conidia, germinating conidia, and protoperithecia are more susceptible to mutation than old conidia when a nitrogen mustard is used as a mutagen. As suggested in the paper the results can be explained by assuming a higher susceptibility to mutation of actively growing and dividing nuclei but they seem to be understandable also on the basis of differences in viability, with a better recovery of mutants from the younger, more vigorous material. The first explanation is certainly reasonable but further experimental support is highly desirable.

Although it has not been possible to produce particular mutations at will by any method, two procedures have appeared that greatly simplify the original process of selecting particular mutants that have occurred in a large population of treated nuclei. Lein *et al.* (1948) described a method of visual selection of mutants following germination of ascospores. More recently Tatum *et al.* (1949) described a method for production of colonial growth in Neurospora by use of sorbose and other specific inhibitors of mycelial growth. This procedure enables selection of mutants through the direct plating techniques used with other organisms. The mononucleate microconidia of Neurospora are used to advantage in this method.

The visual method has the disadvantage that some mutants grow enough following germination of ascospores on the minimal medium so that they are difficult to distinguish from wild type. On the other hand the plating method apparently has the disadvantages of relative inviability of microconidia (Lein and Mitchell, 1948, unpublished) and possible elimination of some mutants by the inhibitors used for production of colonial growth. Both procedures eliminate a large part of the labor involved in the method for isolation of Neurospora mutants originally described (Beadle and Tatum, 1945). Both methods are obviously useful on this basis alone but their relative efficiencies cannot be adequately evaluated at the present time.

A tabulation of the vitamin-requiring mutants that have been obtained is presented in Table I. The data in the table are probably not complete since mutants obtained in different laboratories have not all been compared as to genetic types. It is rather surprising that fewer mutants with vitamin requirements have appeared than have mutants with requirements for amino acids, purines, and pyrimidines. Since the vitamins are chemically complex it would be expected that their synthesis require many biochemical steps and hence many genes. This may be in part a technical matter due to failure to free minimal media from traces of the vitamins. The use of agents such as silica gel for solidifying medium should alleviate this difficulty since this material can be freed from interfering traces of growth substances that are required in very small amounts. Such experiments are in progress in the author's laboratory. There are, no doubt, other more complex reasons for the relative dearth of vitamin mutants.

TABLE I

Vitamin Mutants of Neurospora (B complex)

Vitamin required	Number of different genetic types	Reference
p-Aminobenzoic acid	1	Tatum and Beadle, 1942
Biotin	Wild type	Tatum and Beadle, 1942
Choline	2	Horowitz *et al.*, 1945
Inositol	1	Beadle, 1944
Niacin*	5	Bonner *et al.*, 1943
Pantothenic acid	1	Beadle and Tatum, 1945
Pyridoxine	2	Houlahan *et al.*, 1949; Strauss, 1949
Riboflavin	1	Mitchell and Houlahan, 1946
Thiamine	4	Tatum and Bell, 1946

* For related mutants see Fig. 3 and Table II.

2. Selection of Mutants

The degree of selection that occurs in the process of production and isolation of mutants is of considerable interest in connection with the previous discussion. That is, what proportion of the mutants occurring in treated material are actually obtained as useful cultures? An exact answer to this question cannot be given but an experiment described recently (Lein *et al.*, 1948) may perhaps reveal the order of magnitude of this selection. Using the method of visual observation of mutants produced by ultraviolet radiation, 500 germinated mutant spores were isolated. One half of this number (250) failed to develop past the first germination stage on a medium containing Neurospora autolyzate. (Essentially all of the growth factors required by the mold should be present in this medium.) These mutations are thus lethals which are not even partially repaired by addition of a complex mixture of growth substances. As demonstrated (Lein *et al.*, 1948) some of the mutants in this group can be induced to grow in the minimal medium plus a specific compound. These mutants are evidently inhibited by substances in a complex medium. Besides this type the large group (250) must also include mutants with growth requirements for substances that will not penetrate the cell membrane and thus cannot be supplied from external sources. In addition to requirements for macromolecules such as proteins and nucleic acids, vitamin conjugates and many phosphorylated compounds must be included. For example, growth of Neurospora is not initiated by fructose phosphates as carbon sources while fructose is very satisfactory. Also pantothenic acid mutants do not utilize coenzyme A. The other large group of 215 mutants grow slowly on a minimal or a complex medium. These are clearly mutants of some kind but they have not been the object of extensive investigations. The third and smallest group (35) from the 500 mutants isolated are representatives of the types of mutants that have received the greatest attention in studies of metabolism. Although these figures are approximations at best, duplication in isolations does not appear to be a contributing factor since very little duplication was encountered in the group of identified mutants. Some of the mutants in the "lethal" group of 250 may have been destroyed in isolation but controls indicate that less than 20% are lost in this fashion. The above data demonstrate clearly that the mutants isolated by usual procedures represent a minority (probably less than 10%) of those that occur as the result of ultraviolet treatment of Neurospora conidia. The proportion of identified mutants is probably much less than indicated above since the criterion of observation used in the experiment described is based only on growth.

3. Some Physiological Results of Mutations

It has already been indicated that mutation often leads to a new nutritional requirement through a block in synthesis of some essential metabolite. No less significant however is the effect of this block on the metabolic pattern even though the mutant may be one that appears to grow in a normal fashion when supplied an appropriate growth substance. On both logical and experimental grounds it is becoming more and more obvious that metabolic patterns in mutants are very different from those in the parent wild type strains. Extreme and easily observed accumulations of apparently abnormal by-products are examples (purple pigments, Mitchell and Houlahan, 1946; orotic acid, Mitchell *et al.*, 1948a). Evidently, blocking any major stream of material normally passing through a series of reactions results in either shunting the material into abnormal pathways as indicated above or occasionally in the accumulation of intermediates (Tatum *et al.*, 1944; Horowitz, 1946, 1947; Bonner and Beadle, 1946; Haskins and Mitchell, 1949). For want of criteria for detection fewer cases of accumulations by "shunting" have been described. The accumulation of quinolinic acid (Bonner and Yanofsky, 1949) and kynurenic acid (Haskins, 1949, unpublished) by niacin mutants appear to fall in this category. Several other examples have been found recently (Mitchell and Mitchell, 1949, unpublished). The accumulated substances may represent metabolic "dead ends" but they may also be substances that are formed faster than they are used. These phenomena are evidently similar to those described under "shunt metabolism" by Foster (1949). Here the alterations in metabolic patterns result from environmental changes but such responses to changes in environment are surely reflections of the genetic constitution of the organism In the work with the Neurospora mutants the environment is held constant as nearly as possible.

Tentatively included under the heading of physiological results of mutations, are the special properties of pH and temperature sensitivity. Stokes *et al.* (1943) described the first of these, a mutant requiring pyridoxin at pH 5.0 but having the capacity to synthesize a quantity of the vitamin adequate for growth above pH 6.0. Strauss (1949) has recently shown that the pyridoxine requirement of this and similar mutants is inversely dependent on the concentration of free NH_3 in the medium. By increasing the NH_4^+ (and thus NH_3) concentration, the mutants will grow in the absence of pyridoxine at lower pH values than 5.0. Further elucidation of the nature of this phenomenon can be expected in the near future. Several of the temperature-sensitive mutants of Neurospora have now been described. Among the vitamin mutants, strains with requirements for riboflavin (Mitchell and Houlahan, 1946a) and inositol (Beadle, 1945, unpublished) are represented. In these two cases as in most, the growth factor is required at 35 but not at 25°C. One strain has been found in which the situation is reversed. Twenty-six temperature mutants have been found (Houlahan and Mitchell, 1947, unpublished) and 12 of these can be induced to grow when supplied with the appropriate nutrient. The remaining 14 strains evidently would have fallen in the "lethal" category already discussed if they had not been temperature-sensitive mutants. The suggestion was made (Mitchell and Houlahan, 1945) and more recently expanded (Horowitz, 1950) that the ratios of these two kinds of temperature mutants might be an index of the degree of selection encountered in isolation of mutants of Neurospora. As already discussed the degree of selection appears to be much higher than that indicated by this ratio.

For the present both the pH and temperature mutants may be considered as resulting from special kinds of partial genetic blocks. Indeed, evidence has been presented to show that the same gene is involved in two kinds of temperature mutants and a nontemperature sensitive mutant (Houlahan and Mitchell, 1947; Mitchell and Houlahan, 1947). It seems likely that these phenomena are related to both the nature of mutation and the effects of the mutation on the metabolic pattern of the mutants. It is also significant that changes in genetic constitution can result in different degrees of nutritional requirements as well as in absolute requirements.

4. Genetic Analyses

The technical advantages in the use of Neurospora for investigations in genetics have been discussed extensively elsewhere (Dodge, 1927; Lindegren, 1942; Beadle, 1945b). Work of McClintock (1945) and Singleton (1948) has contributed extensively to knowledge of the morphology of the chromosomes of Neurospora and has provided a foundation for further work in cytogenetics with this mold. Although all of the known linkage groups cannot yet be assigned to particular chromosomes a recent report (Houlahan *et al.*, 1949) has presented a summary of genetic analyses of about 69 mutants showing approximate loci of 35 genes on five of the seven chromosomes of the mold. Twelve of these are different genes concerned with six of the B complex vitamins. Data at present are far from sufficient to provide evidence on the interesting question as to whether the location of these genes on the chromosomes has a significant relation to metabolic control. Such position effects have been established in some cases in other organisms (Lewis, 1950).

III. GENES, ENZYMES AND REACTIONS

1. The Gene-Enzyme Hypothesis

Clearly one of the problems of greatest significance in studies of metabolism is concerned with the inheritance of biochemical capacities. Beadle in 1946 presented the simple hypothesis that each gene is concerned with conferring final specificity on one enzyme, which then in turn catalyzes a particular reaction or type of reaction. The hypothesis was derived directly from investigations of Neurospora mutants, although similar but less specific suggestions had been made previously from knowledge of genetics and metabolism in other organisms (see Beadle, 1946). In spite of much discussion pro and con there is little question but that this hypothesis has and will continue to serve as a basic guiding principle in investigations in biochemical genetics. It is also clear, however, that modifications and revisions will be necessary as experimental work progresses. For example the hypothesis was originally based on studies of the classified mutants, the smallest group (7%) in the example already cited. Mutants of the two large classes may act in a different way and require a revision of the hypothesis. This is at best a negative sort of criticism. There are a number of other points which would appear to be of more fundamental importance. The first is concerned with the gene itself. The hypothesis implies that the gene, the enzyme and the reaction can be defined in the same units. That is, in terms of molecules acting in accordance with known chemical and physical laws. In spite of the rapidly increasing knowledge of the chemical nature of chromosomes and macromolecules of living cells it is still impossible to define a gene as a chemical substance. It remains a unit of inheritance with knowledge of its existence dependent on observations of its effects on an organism. It is quite conceivable that the gene can be defined eventually in biochemical terms but it is most difficult to visualize what it does without knowledge of what it is. With respect to the vitamins, no information at all is available as to the part they play in the processes of gene reproduction and gene action. Since the vitamins are known to play such a significant role in catalyses by enzymes it will be indeed surprising if they are not also found to be involved in some of the catalytic functions of genes.

In the same vein it is not inconceivable that the vitamins have a part in the process of mutation. Some of the vitamins, niacin for example, have absorption spectra in ultraviolet light that correspond about as well to mutation action spectra (see Beadle, 1945a, for review) as do the nucleic acids. On the other hand so do a number of other compounds. In any case it is now clear that mutations in genes can be caused by various reactive chemicals as mentioned previously, as well as by direct action of radiant energy. Radiation may indeed act indirectly through activating some simple molecules at the right place and the right time to cause mutations. In the opinion of the author there is little justification at present for considering any one kind of chemical agent to be the significant type of substance in causing mutations. Any substance that can penetrate to the genes, without killing the cell, and can react with functional groups of genic substances, is certainly a potential mutagen.

2. *Enzymes*

In considering the problems of biochemical genetics at the enzyme level, it is possible to speak more in terms of molecules, coenzymes and chemical kinetics. Complications arise nevertheless from the problem of what might be called "biochemical morphology." It is now evident that, in at least some cases, a group of enzymes may exist in the cell in a conglomerate and that in this state the enzymes may act more effectively than they do when studied individually. In its fundamentals there is no obvious reason for considering this type of morphology to be different from that resulting from differentiation in a complex organism.

Certainly these phenomena must at some time be woven into the biochemical pattern describing the relations of biochemistry and genetics.

Recent investigation on chromatography of some enzymes on filter paper (Mitchell *et al.*, 1949) have given rise to the suggestion that at least some enzymes may consist of a complex mixture of different molecular species of proteins. Such a conclusion is far from justified on several counts but further investigation should clarify the question. It is quite possible that the apparent multiplicity of components in the enzymes described is due to associations or to partial denaturation of the enzymes in the processes of extraction. Unpublished results in the author's laboratory have indicated a multiplicity of components in crystalline sweet potato amylase.

Investigations have been reported on three cases in Neurospora in which genetic constitution can apparently be related directly to a biochemical reaction through an enzyme system that can be studied *in vitro*. The first of these, the conversion of adenosine to inosine, is not known to involve any of the vitamins although an unknown coenzyme may be involved in the reverse reaction. Since the latter has not been accomplished *in vitro* the investigation established only that the enzyme system for deamination as obtained from wild type and a mutant were indistinguishable by the criteria used (McElroy and Mitchell, 1946). Investigations of particulate enzyme systems may provide more informative data on this system.

More recently two systems, both involving vitamins, have been investigated and related directly to particular gene mutations in Neurospora. The first case, the synthesis of pantothenic acid, has been investigated by Wagner and Guirard (1948) and Wagner (1949) at the University of Texas. Here it was shown that a mutant requiring pantothenic acid and unable to use pantoyl lactone plus β-alanine is unable to synthesize the vitamin from the two fragments (Fig. 1). Washed mycelium, ground mycelium and acetone powders were used. Under the same conditions pantothenic acid synthesis occurred using wild type strains of Neurospora. Thus it appeared that an enzyme activity was missing as a result of a single gene mutation in the mold. It was found, however, that enzyme activity appeared in the mutant preparations when the insoluble material (containing the enzyme) was washed thoroughly with water. Thus it became clear that in this case the mutation did not result in the loss of an enzyme but resulted in its inability to function *in vivo*. The exact mechanism of this inactivation remains to be determined though a number of possibilities are evident.

The second case illustrated in Fig. 1 was investigated following the isolation of the mutant concerned with this particular reaction by means of the visual method of selection already discussed (Lein *et al.*, 1948). Following the methods of Umbreit *et al.* (1946), who originally described the enzymatic synthesis of tryptophan with preparations from Neurospora in the presence of pyridoxal phosphate, it was shown that cell-free extracts of mutant C-83 failed to carry out the reaction while corresponding wild type extracts gave excellent tryptophan synthesis (Mitchell and Lein, 1948). Subsequent work (Gordon and Mitchell, 1949, unpublished) indicated that purification of the enzyme preparations from the mutant resulted in the appearance of an active tryptophan-synthesizing system. Thus, the case appeared to be similar to the one concerned with pantothenic acid synthesis, but recent work has cast doubt on this conclusion. In the most recent work with strains of C-83 of known genetic purity with respect to the tryptophan requirement, the tryptophan-synthesizing system has not been found in numerous experiments under various conditions. Further investigations are required to clarify this important point. Obviously it is not possible, by ordinary means, to prove the absence of the enzyme.

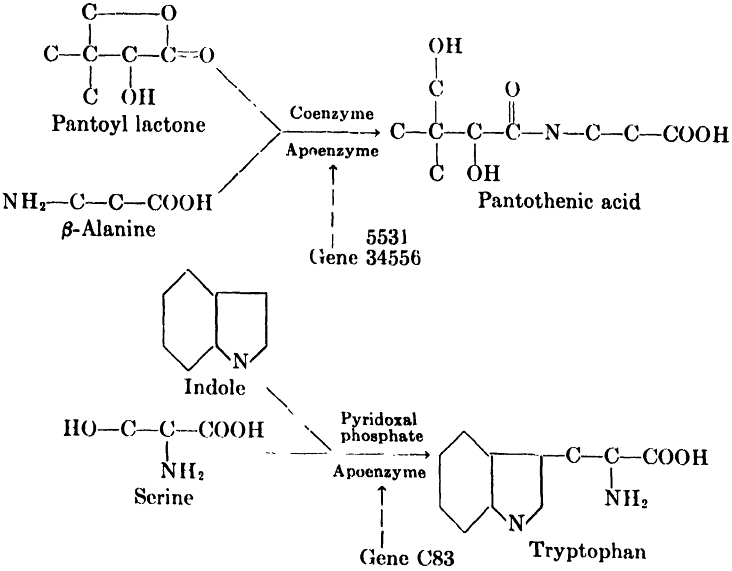

FIG. 1. Reactions and enzyme systems in Neurospora related by *in vitro* studies to particular gene mutations.

It should be pointed out clearly that both of the mutants just discussed have absolute requirements for pantothenic acid and tryptophan, respectively. In neither case is there any indication of a "partial block" in synthetic ability. Thus, these mutants fall in the small group of classified mutants mentioned earlier and not in the large group considered to carry partial genetic blocks. Even so, it is not certain that mutation results in a loss of an enzyme as a chemical entity. Thus, like the gene, a corresponding enzyme is recognized at present by its effect and not by its chemical nature.

IV. Biosynthesis and Functions of Vitamins

1. *p-Aminobenzoic Acid*

Although a mutant requiring PABA was one of the earliest reported of the Neurospora vitamin mutants (Tatum and Beadle, 1942) a recent investigation by Tatum (1949) provides the first evidence as to the biological origin of this substance. It was shown that a Neurospora mutant required a mixture of PABA, anthranilic acid, phenylalanine, and tyrosine. The conclusion that these substances arise from a common precursor is in accord with, and extends the report from this laboratory (Nyc *et al.*, 1949a) that the aromatic amino acids have a common origin. These relationships are indicated in Fig. 3. A good many intermediates are to be expected since these four aromatic compounds are not obviously closely related.

Thus far the mechanism of action of *p*-aminobenzoic acid in Neurospora remains obscure. The PABA mutants do not utilize folic acid and since no folic acid mutants have been found there is no evidence that PABA functions only after conversion to folic acid or related compounds.

The relation of sulfonamide inhibition to PABA utilization in Neurospora has received considerable attention since the first paper on a PABA mutant (Tatum and Beadle, 1942). An early report provided information that sulfanilamide has only an indirect effect on respiration in the mold. (Giese and Tatum, 1945.) More recently Emerson and coworkers have presented a series of papers concerned with sulfonamide-PABA relations. By means of selection of spontaneous mutants, a strain of Neurospora was obtained which is resistant to sulfonamides and another was obtained which requires sulfonamides for growth under certain environmental conditions (Emerson and Cushing, 1946). Changes in single genes were shown to be involved. Growth of the sulfonamide-requiring strain was shown to be inhibited by PABA. Detailed studies on growth characteristics of these strains were published by Emerson (1947) and subsequently by Zalokar (1948). Conclusions drawn from experiments by the latter author suggested that the sulfonamide requirement is due to production of an inhibitory amount of PABA by the mutant and that the sulfonamide merely relieves this inhibition and is not, in itself, a metabolite. This conclusion was derived from the fact that the double mutant carrying both the PABA requirement and sulfonamide requirement grows with low concentrations of PABA but becomes inhibited at higher concentrations. This inhibition is then relieved by sulfonamides, hence the requirement. The conclusions seem entirely reasonable but unfortunately are not accompanied by analytical data on the quantity of PABA produced by the sulfonamide requiring mutant. Limitation of PABA production as with the double mutant does not necessarily provide a complete picture since the limitation of PABA by the mutation may not be complete. In addition introduction of the PABA requirement may cause changes in the metabolic pattern not present in the sulfonamide requiring strain. Indeed, recent evidence showing that mutations in many genes cause a disappearance of the sulfonamide requirement (Emerson, 1948) implies that the situation is more complicated than was considered previously (Zalokar, 1948). Some further complications have been discussed recently by Emerson (1950). It has been shown that a sulfonamide-methionine double mutant acts in the same fashion as the sulfonamide-PABA double mutant. It might be expected that introduction of adenine requiring mutants will have similar effects since adenine as well as methionine has been shown to alleviate sulfonamide inhibition (Emerson, 1949). It seems likely that growth studies will not yield an entirely satisfactory answer to the sulfonamide requirement. Investigations of appropriate enzyme systems *in vitro* should be more satisfactory but knowledge of the role of PABA in metabolism is insufficient to make this approach practicable at the present time.

2. Biotin

Wild-type Neurospora requires biotin for growth and hence its use in studies of biosynthesis of this vitamin is limited. Tatum (1945) reported utilization of desthiobiotin by Neurospora and, since this substance appears to be a precursor to biotin in *Penicillium chrysogenum*, it may be also in Neurospora. Pimelic acid however is inactive.

No information is available on the mechanism of action of biotin in the mold. Recently a biotin conjugate (biocytin) has been isolated from yeast and has been shown to replace biotin for the growth of Neurospora (Wright *et al.*, 1949). Whether this conjugate is utilized as a coenzyme in the mold remains to be established.

3. Choline

Several steps in biosynthesis of choline in Neurospora have become clear from investigations by Horowitz (Horowitz *et al.*, 1945a; 1946).

The known series of reactions is indicated in Fig. 2. Monomethylethanolamine was shown by isolation, to be accumulated by strain 47904. This substance as well as the dimethyl derivative is utilized by strain 34486 while both strains utilize choline. Strain 47904 carries a partial block in choline synthesis and provides an interesting case in which a mutant accumulates a substance that it can use if the compound is given in a high concentration. Evidently the mutation simply reduces the rate of methylation of monomethylaminoethanol to the dimethyl compound so that the former compound is made faster than it is utilized. A considerable number of compounds besides choline and dimethylaminoethanol will satisfy the growth requirements of both of these mutants (Horowitz *et al.*, 1945a). These include acetylcholine, arsenocholine, phosphorylcholine, dimethylethylhydroxyethyl ammonium chloride, triethylcholine and methionine. Utilization of methionine by both mutants is rather surprising and is perhaps best explained as a sparing action provided it is assumed that both mutants have the ability for partial synthesis of choline. The latter appears to be the case though no analytical data are available.

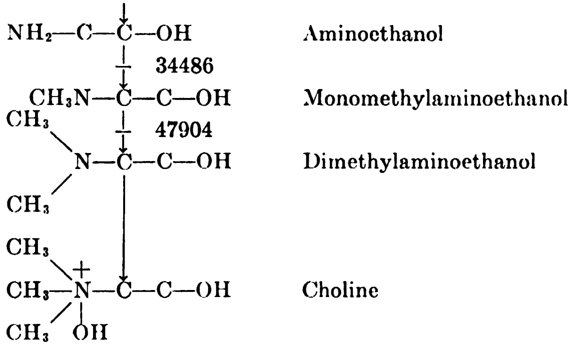

Fig. 2. Biosynthesis of choline.

Conjugated forms of choline in Neurospora have not been investigated. Nor has evidence of its participation in transmethylation reactions been demonstrated directly with enzyme systems.

4. Inositol

Beadle (1944) has reported on inositol requiring mutants of Neurospora and has developed a bioassay for this vitamin. Only one genetic type of mutant is available and this does not use scyllo-inosose or inositol monophosphate (Mitchell, 1947, unpublished). Information on conjugates and action of inositol in metabolism is entirelylacking.

5. *Niacin*

The mechanism of biosynthesis of niacin in Neurospora has received considerable attention and it is now possible to describe a complex and extensive series of reactions leading to the biological formation of this vitamin. The picture as it stands at present is shown in Fig. 3. In the scheme shown the arrows do not necessarily represent single step reactions but indicate only the order of the various intermediates indicated. The cross bars and corresponding numbers indicate different mutant strains that have been investigated. Evidence for the common origin of the benzene nucleus has been presented from the author's laboratory (Nyc *et al.*, 1949a) and by Tatum (1949), the latter including *p*-aminobenzoic acid in the picture as discussed previously. Obviously a considerable number of intermediates are involved in the formation of a ring and its conversion to indole or anthranilic acid. The portion of the cycle shown extending from anthranilic acid through tryptophan was originally established by Tatum *et al.* (1943). Subsequently it was shown that tryptophan is converted to kynurenine by the mold (Beadle *et al.*, 1947) and recently Haskins and Mitchell (1949) provided evidence for the existence of the cycle shown in Fig. 3. This evidence rests on a number of experimental facts. First, anthranilic acid is produced very rapidly from tryptophan by germinating conidia of mutants and wild type alike. At least 25% of the tryptophan disappears from the cultures by the time 1% of maximum growth is produced. During subsequent growth the anthranilic acid disappears and tryptophan and niacin are produced. Indole and kynurenine, like tryptophan, serve as precursors of the anthranilic acid produced by germinating conidia. On the other hand strains 10575 and C-83 do not utilize kynurenine for growth and the reaction tryptophan → kynurenine is therefore irreversible. Thus, the evidence suggests the conversion of anthranilic acid to tryptophan via a different pathway from that of its production from tryptophan. Mutants 10575 and C-83, concerned with reactions falling within the cycle, grow poorly as might be expected. Strain 39401 grows well but has some unusual properties that will be discussed subsequently.

Steps in the conversion of kynurenine to niacin have been established in a number of ways. Hydroxyanthranilic acid was synthesized as a possible intermediate in this series of reactions (Nyc and Mitchell, 1948) and was shown to have the appropriate biological activity for the Neurospora mutants (Mitchell and Nyc, 1948). The intermediate accumulated by strain 4540 (Bonner and Beadle, 1946) was then established as being identical with 3-hydroxyanthranilic acid (Bonner, 1948). In a paper in 1948 hydroxykynurenine was proposed as an intermediate

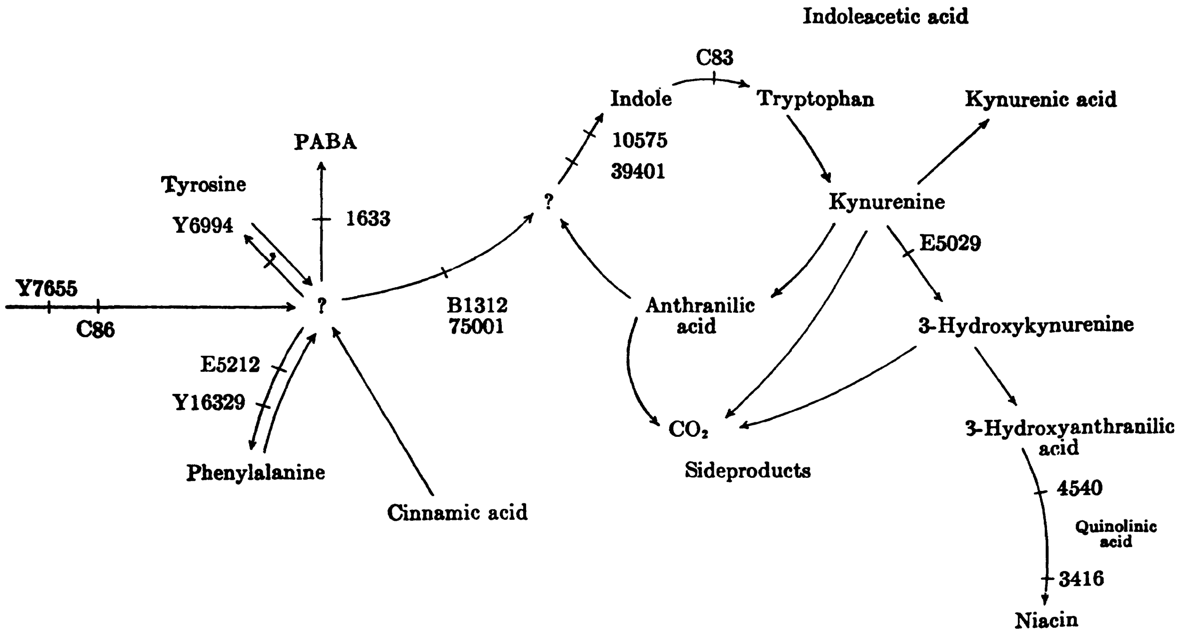

Fig. 3. Biosynthesis of niacin. Cross bars and numbers represent different Neurospora mutants.

between kynurenine and hydroxyanthranilic acid (Mitchell and Nyc, 1948). The proposal was confirmed recently in this laboratory (Haskins and Mitchell, 1949) when the compound became available through the kindness of Dr. W. Weidel of the Kaiser Wilhelm Institut für Biochemie.

The substance was first isolated from the larvae of *Calliphora erythrocephala* in 1949 by Dr. Weidel, in a continuation of the extensive investigations on insect eye pigments, directed by Prof. A. Butenandt at the Kaiser-Wilhelm-Institut für Biochemie.

In order to clarify further the picture presented in Fig. 3, the relations between the compounds and the mutants used are summarized in Table II. Two of the mutants listed in the table (C-86 and 39401) require special consideration. Since the early work with strain 39401 (Beadle *et al.*, 1947) it has been known that, in spite of the fact that this mutant does not grow on minimal medium, it requires 400 μg./20 ml. of tryptophan for maximum growth but only 15 μg./20 ml. of niacinamide. Clearly the tryptophan requirement of this strain is satisfied by means other than reversal of the tryptophan to niacin series of reactions. The picture is similar with strain C-86 although this mutant grows to a slight extent on minimal medium. This phenomenon now appears to be a result of partial losses in capacity to carry out particular reactions. In both cases the mutants evidently possess the ability to synthesize considerable quantities of tryptophan but not enough to satisfy all of the side reactions of the cycle as well as to supply the material for niacin synthesis. As pointed out recently (Haskins and Mitchell, 1949) anthranilic acid is produced by the mold at least 50 times as fast as niacin thus providing adequate means for dissipation of large quantities of tryptophan with production of only very small quantities of niacin. Some of the known side reactions of the cycle are shown in Fig. 3.

The above interpretation would appear to have considerably greater significance than as an explanation of the properties of these two particular mutants. In view of the findings already discussed in which it has been shown that mutation has not resulted in the loss of an enzyme in both of the cases investigated it seems likely that partial synthetic ability is common even among mutants with apparent absolute requirements. Mutants that obviously fall in this category (pH and temperature mutants) have already been discussed. A real understanding of this phenomenon must await further knowledge of the biochemical relations between genes and enzymes.

Some use has been made of tracers in investigations of reactions in Neurospora concerned with the biosynthesis of niacin. Anthranilic acid containing C^{14} in the carboxyl group was given to a mutant that is similar in its growth requirements to strain B-1312 (Nyc *et al.*, 1949b) (Fig. 3).

TABLE II

Mutants Requiring Niacin and Metabolically Related Substances

	1633	E5212	Y6994	75001 B1312	C86	39401	10575	C83	Y7655*	E5029	4540	3416
PABA	+	−	−	−	−	−	−	−	+	−	−	−
Phenylalanine	−	+	−	−	+	−	−	−	+	−	−	−
Tyrosine	−	−	+	−	+	−	−	−	+	−	−	−
Cinnamic acid	−	−	−	−	+	−	−	−		−	−	−
Indole	−	−	−	+	+	+	+	−		−	−	−
Tryptophan	−	−	−	+	+	+	+	+		−	−	−
Kynurenine	−	−	−	+	+	+	−	−		−	−	−
Anthranilic acid	−	−	−	+	+	−	−	−	+	−	−	−
Hydroxykynurenine	−	−	−	−	+	+	−	−		+	−	−
Hydroxyanthranilic acid	−	−	−	−	+	+	−	−		+	−	−
Niacin	−	−	−	−	+	+	−	−		+	+	+
Niacinamide	−	−	−	−	+	+	−	−		+	+	+

* This strain requires all four compounds listed. Substitutes for any components have not been reported although it would be expected that indole, tryptophan, or kynurenine will replace the anthranilic acid. Quinolinic acid is not included although it is reported to have some growth promoting activity for 4540 but not 3416. Kynurenic acid and indoleacetic acid do not support growth of any of the mutants.

Tryptophan and niacin were isolated from the mold after growth. Neither contained radioactivity. All of the C^{14} was found in CO_2 given off during growth of the culture. Evidently the carboxyl group of anthranilic acid is exchanged for some other group during conversion to indole. Other mechanisms are also possible but this appears the most likely place in the cycle for loss of the carboxyl group carbon atom. Experiments have been described recently (Leifer *et al.*, 1950) in which strain C-86 was provided with 3-hydroxyanthranilic acid containing N^{14} in a medium containing N^{15} as NH_4^+. It was calculated that 49 and 43% of the niacin nitrogen, from two independent experiments, was derived from the ammonia in the medium. In view of the evident partial ability for tryptophan synthesis in strain C-86, interpretation of this result is uncertain. However, since the requirement for niacin by C-86 is not significantly less than that of other mutants such as 3416 and 4540 it seems unlikely that the niacin N^{15} represents the amount of leakage. A symmetrical intermediate containing two atoms of nitrogen is suggested. It is not clear how this is related to accumulation of quinolinic acid by strain 3416 (Bonner and Yanofsky, 1949; Henderson, 1949). Bonner has proposed a simple scheme for conversion of hydroxyanthranilic acid to niacin with quinolinic acid as a byproduct with very slight activity. 3,4-Dihydroxyanthranilic acid might be expected to be an intermediate by this scheme but this compound has been shown to be inactive (Mitchell, 1948, unpublished). On the other hand Henderson (1949) considers quinolinic acid to be an intermediate with low activity due to nonpenetration of the cell by salts of the strong acid. Further work is required to justify any of these three different points of view.

Accumulation of an inorganic polyphosphate by strain 39401 (Houlahan and Mitchell, 1948) may well be a reflection of abnormal coenzyme functions. The substance is accumulated in large quantities when the mutant is grown with limiting amounts of indole, tryptophan, or kynurenine (Fig. 3) but not when it is grown in the presence of hydroxyanthranilic acid or niacin. It appears that the polyphosphate accumulates when the cycle fails to function in the presence of substances that contribute directly to its function.

Coenzymes containing niacin itself have not been investigated in Neurospora.

It is inappropriate here to discuss niacin biosynthesis in other organisms but it should be pointed out that various parts of the total scheme in Fig. 3 have been shown to take place in many other organisms, including mammals (Snell, 1943; Ellinger, A., 1904; Rosen *et al.*, 1946; Mitchell *et al.*, 1948b; Albert *et al.*, 1948; Heidelberger, 1949; Heidelberger *et al.*, 1949a and b; Schweigert, 1949). However, the cycle described is operative in Neurospora only, so far as is known at present.

6. *Pantothenic Acid*

As already discussed pantothenic acid is produced in Neurospora by an enzymatic coupling of β-alanine and pantoyl lactone (Wagner, 1949). Although Tatum and Beadle (1945) provided evidence for accumulation of these two substances by a pantothenic acid mutant, their origin in the mold is unknown. Conjugates of pantothenic acid in the mold have not been investigated though it has been shown that the Neurospora mutants do not utilize the pantothenic acid-containing coenzyme A of Lipmann (Mitchell, 1949, unpublished).

7. *Pyridoxine*

Although pyridoxine-requiring mutants were among the first produced nothing has been reported as to the mechanism of biosynthesis. Pyridoxine, pyridoxal, and pyridoxamine are utilized equally well by the Neurospora mutants (Beadle, 1946, unpublished). Strauss (1949) has recently reinvestigated the pH sensitivity of one of the pyridoxine mutants previously investigated by Stokes *et al.* (1943). The pH sensitivity has been correlated closely with the concentration of non-ionized NH_3 in the culture medium. Pyridoxine is not required when the concentration of NH_3 is sufficiently high.

A partial block in pyridoxine synthesis with a resulting upset in the metabolic pattern may contribute to the phenomenon.

As already pointed out, pyridoxal phosphate (Merck, synthetic) is involved in the coupling of indole and serine. Other coenzyme actions of pyridoxine derivatives in the mold have not been reported.

8. *Riboflavin*

The one riboflavin mutant of Neurospora that has been reported is temperature sensitive and has been shown to produce riboflavin even at temperatures at which it is required. (Mitchell and Houlahan, 1946a.) Several compounds were tested as intermediates but none promoted growth of the mutant. Lumichrome and lumiflavin were found to be competitive inhibitors.

9. *Thiamine*

Investigations on the mechanism of the biosynthesis of thiamine have not been reported since the paper of Tatum and Bell (1946). The data indicated that thiamine is formed in Neurospora as indicated in Fig. 4. Accumulation of a pyrimidine component by mutant 18558 and of both pyrimidine and thiazole components by 9185 was shown by bioassays. These accumulations are in reasonable agreement with the scheme presented. Two other mutants 1090 and 17084 do not fit satisfactorily into the simple picture since both require either intact thiamine or a pyrimidine-thiazole mixture. In addition 17084 accumulates a substance with thiamine activity for strains 9185 and 18558. The suggestion was made that strains 1090 and 17084 carry partial blocks in the utilization of thiamine since both have especially high requirements for the vitamin. This possibility is described in Fig. 4. Isolation of the substance accumulated by 17084 should do much to clarify this picture. It would seem to be in order also to isolate the substances accumulated by 9185 and 18558. Bioassays are certainly inadequate for identification of the presumed intermediates. For example it seems unlikely that the natural pyrimidine fragment contains a 5-ethoxy group corresponding to the synthetic compound used for testing. A reinvestigation of this problem would be of considerable interest.

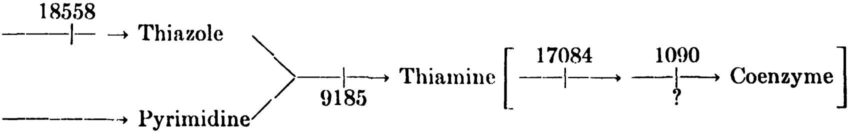

FIG. 4. Biosynthesis of thiamine. Cross bars and numbers represent different Neurospora mutants.

Cocarboxylase is evidently utilized by Neurospora (Tatum and Bell, 1946) but the nature of thiamine conjugates in the mold is unknown.

10. Other Vitamins

Requirements have not been demonstrated in Neurospora for ascorbic acid or any of the fat soluble vitamins. Zechmeister and Haxo (1946) and Haxo (1949) have, however, investigated the pigments of wild-type Neurospora and have demonstrated the presence of β-carotene, γ-carotene, spirilloxanthin, and lycopene as well as a new substance given the name of neurosporene. Three other polyene pigments were isolated but were less well characterized. The colorless but fluorescent polyene, phytofluene was also obtained in considerable quantities. Pigment production is increased in the light but both pigments and phytofluene are produced in the dark. No mention was made of vitamin A.

Although these carotenoids are not required for growth, investigations of the presence of various of the compounds in partial albino and in albino mutants of Neurospora (Hungate, 1946) should yield interesting information on carotenoid biosynthesis.

11. Unknowns

Although mutants with unknown requirements have been available for a considerable period of time these have not been investigated systematically. A few have been found to have double requirements such as isoleucine plus valine (Bonner *et al.*, 1943) and adenine plus methionine (Lein *et al.*, 1948). A program is in progress in the author's laboratory for investigation and production of mutants requiring unknown substances. Eight mutants are available at the present time.

V. BIOASSAYS

Presumably any of the Neurospora mutants can be used in bioassay for the substances required. There are, however, certain aspects of this problem that should be considered here. It should be noted that while wild-type Neurospora grows quite satisfactorily on a synthetic medium, its growth can be approximately doubled by addition of materials such as yeast or liver extracts. This is evidently due to nonspecific sparing actions by substances in the extracts. In any case the picture is similar with mutants. An accurate bioassay using synthetic media cannot be expected unless the substance being measured is present in sufficient amounts so that supplementary effects can be diluted out.

A second question of significance in the use of artificially induced mutants for bioassays arises from occasional serious disturbances in metabolism that occur as a result of a single gene mutation. The thiamine mutant 9185 has been shown to be inhibited in a yeast extract medium (Houlahan and Beadle, 1945, unpublished) with, in this case, a selection for back mutation resulting. Other more extreme cases of inhibition by natural extracts have been described recently (Lein *et al.*, 1948). In the natural environment it is to be expected that spontaneous mutants of this kind will be eliminated unless the unfavorable effect of the mutation is removed by back mutation or through a second mutation. An example of the second possibility has been described recently in Neurospora (Mitchell and Mitchell, 1950). From a consideration of these facts it seems a reasonable conclusion that natural selection probably gives rise to strains of microorganisms that are more favorable for bioassays than do induced single-gene mutations. This, like many generalizations, must have exceptions and the problems with the induced mutants are sometimes also encountered with naturally occurring strains with particular requirements.

In spite of the above and other less significant factors a number of bioassays have been developed using the Neurospora mutants. One of the most useful is that for choline (Horowitz and Beadle, 1943) since few specific analytical method are available for determinations of this vitamin. In this case partial purification of the vitamin is used as a preliminary step to bioassay, thus eliminating the problems of supplementation and inhibition.

An assay for PABA was developed (Mitchell *et al.*, 1943; Thompson *et al.*, 1943) using a complex medium to alleviate supplementary effects. Surface growth on a solid medium was used to provide a rapid assay procedure requiring 16 hours rather than the 3-day minimum for liquid culture assays. The method is convenient for purposes such as the following of isolation procedures but it is less accurate than the liquid culture techniques used in most assays with Neurospora and it is unsatisfactory with some mutants.

An apparently satisfactory assay for inositol with Neurospora was developed by Beadle (1944). A rigorous comparison with other methods has not been made. Phytin and lipositol are not utilized by the mutant.

The bioassay developed by Stokes *et al.* (1943) for pyridoxine is now known to measure pyridoxal and pyridoxamine as well. The method appears satisfactory for measuring these three substances simultaneously. Tatum *et al.* (1946) has reported use of Neurospora in assays for biotin, choline, inositol, PABA and pyridoxine, all in synthetic media. Accuracy of these procedures for all of the material assayed seems open to some question for reasons previously discussed.

Assays for niacin, pantothenic acid, riboflavin and thiamine using Neurospora mutants have not been reported. From previous discussion it appears that the riboflavin and thiamine mutants have unfavorable characteristics for use in bioassays.

In using the liquid culture assay method, Ryan (1946) has suggested use of glass filters and elaborate precautions for increasing accuracy. These refinements seem unnecessary for most work since variability in growth usually exceeds variations due to the processes of removing and drying mold pads.

Mention should also be made of two techniques for measurement of growth of Neurospora other than those already given. The growth tube procedure of Ryan *et al.* (1943) is useful for direct measurement of growth rates. It is, however, rather too tedious for routine bioassays. Ryan (1948) has developed a method by which very rapid assays can be carried out. The method is based on observations of germination rates and is limited to those mutants that fail to germinate without a particular growth substance. From the author's experience relatively few mutants fall in this category.

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The Physiology of Relaxin

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I. INTRODUCTION

The discovery of relaxin was the outcome of studies concerning pelvic adaptations associated with parturition. The comparative morphologists previously had described the marked differences in pelvic structure that exist among mammals and their correlation with conditions of habitat in which the various species live. In general, those that live in burrows, or are pelagic, or fly, have constricted pelves and consequently a reduction in the diameter of the birth canal. A small pelvis is an obvious advantage to an animal that must turn in the narrow confines of a burrow or whose body is streamlined for swimming or flying, but is a serious disadvantage for the birth of young. Indeed, in certain instances the pelvic cavity is so narrow that delivery would be impossible were it not for special adaptations to meet the situation.

This problem, as one might expect, was not solved in the same way in distantly related species. The most extreme reduction of the pelvis, among burrowing mammals, is found in the mole. Embryos of *Scalopus aquaticus machrinus* (Raf.) 20 mm. in length have pelvic girdles of the usual mammalian type. However, the cartilaginous pubic arch does not become ossified but is resorbed in both males and females by the time the animal is half-grown and the two halves of the pelvis join to form a secondary symphysis dorsal to the digestive tract (Hisaw and Zilley, 1927). Thus in the adult the reproductive tract is entirely outside and ventral to the pelvic girdle which obviates the necessity of parturition through a constricted birth canal. The endocrine system apparently does not take part in bringing about this adaptive arrangement. It must be explained on some other basis, probably genetic.

In certain mammalian species the symphysis pubis reacts to estrogen as a female secondary sexual character, giving rise to a sex dimorphism of the pelvis. The best example of this is probably the pocket gopher, *geomys bursarius* (Shaw). The pelvis of this animal is complete in both male and female and much too small for the birth of young. The pubic cartilages in both sexes become ossified and unite to form a rigid symphysis pubis. With the approach of the first estrus the pubic bones in the female are gradually resorbed leaving the pelvis open ventrally while those in the male remain intact. Absence of the pubic bones makes it possible for parturition to proceed unobstructed (Hisaw, 1925).

Another adaptive mechanism of this sort is one in which there is a sex dimorphism of the pelvis, similar to that in the pocket gopher, but in addition parturition is further facilitated by marked relaxation of the pelvic ligaments. The most extensive modification of this nature occurs in the guinea pig, in which ligamentous relaxation takes place both at the symphysis pubis and sacroiliac junctions (Hisaw, 1929; Ruth, 1937). This condition develops during gestation and becomes so pronounced that in the parturient animal the two halves of the pelvis are widely separated and can be moved freely and independently of each other. Research on the physiology of this reaction has demonstrated that it is under hormonal control. One of the hormones that, in large measure, is responsible for this pelvic modification in the guinea pig has been named relaxin and its properties and actions are the subject of the present discussion.

II. HISTORICAL SURVEY OF PUBIC SEPARATION IN MAMMALS

The phenomenon of "pubic relaxation" or separation of the symphysis pubis in different mammals during pregnancy and parturition has been an established fact for a great many years. Reference to the separation of the "ossa pubis" during labor in women may be found in the writings of Hippocrates. In the eighteenth century two schools of thought arose with reference to symphyseal separation. Mauriceau (1727) denied the concept that pubic bones separated but stated . . . "others are of the opinion that these bones thus separate at the time of labor, are thereto by degrees a little before disposed by the slimy humours which flow forth from the womb and these mollify the cartilage which at other times join them firmly."

1. *Primates*

During the next century a number of anatomical studies were carried out on the pubic symphysis of women, which definitely proved that relaxation occurred. Snelling (1870) indicated that relaxation of the pelvic articulations suddenly became apparent after parturition or gradually during pregnancy, permitting a degree of mobility that not only hindered locomotion but frequently gave rise to alarming and painful sensations. Cantin (1899) examined 500 women and observed that relaxation was marked in the ischiopubic symphysis and much less so in the sacroiliac symphysis. This condition was found only during pregnancy, at which time 98% of the pregnant women showed pelvic relaxation. He further noted that the degree of relaxation increased as pregnancy advanced and that the pubic symphysis returned to normal within a few weeks after parturition.

In 1909 Fick reported relaxation of the pelvic ligaments of pregnant women and postulated that it was caused by a shift in the center of gravity due to the enlarged uterus. Lang and Haslhofer (1932) reported similar observations and described the anatomical and histological changes in the pubic symphysis of women at term and of men following heavy work. They concluded, in agreement with Fick, that the increased symphyseal mobility was caused by mechanical strains and changes of posture. However, these opinions have not been substantiated.

In 1905 Goldthwait and Osgood reported for the first time that not only did pelvic relaxation occur in women during pregnancy but also to a slight degree during menstruation. Lynch (1920) showed marked pubic relaxation during late pregnancy by the use of the X-ray technique. Chamberlain (1930, 1937) measured the degree of pubic separation in pregnant and nonpregnant women by roentgen examination and found that definite relaxation occurred by the fourth week of pregnancy. In addition he observed pelvic relaxation in nonpregnant women complaining of low back pain. Pubic relaxation occurred concomitantly with the pain and varied from 0 to 1.5 mm. of separation. The height of the pain was observed just prior to menstruation.

Other investigators, using the X-ray technique, have confirmed the separation of the pubic bones in pregnant women and closure of the symphysis pubis by 6 weeks post partum (Martius, 1930; Müller, 1931; Boland, 1933). Heyman and Lundquist (1932) measured the width of the pubic symphyses in 74 pregnant women and obtained an average separation of 1.2 cm. The greatest degree of separation was found to appear between the 5th and 7th month of pregnancy. However, no measurements were taken prior to the 5th month. Nevertheless, it is of interest to note that the maximum separation occurred prior to the last two months of pregnancy. Abramson *et al.* (1934) concluded that relaxation of the pelvic joints began in the first half of pregnancy and showed only slight increase during the final three months. Further confirmation of pelvic relaxation was presented by Thoms (1936) who measured by roentgenograms the separation in 100 primiparous women. Twenty-eight per cent of those examined in the first half of pregnancy showed a separation of 2 to 6 mm.; 72% of those examined in the second half of pregnancy showed a spread of 3 to 10 mm. Thoms concluded that relaxation of the pubic symphysis of women occurs in normal pregnancy and is a progressive phenomenon.

Separation of the pubic bones during pregnancy has been observed also in the rhesus monkey (Straus, 1932; Hartman and Straus, 1939). In this species the separation was most marked in the sacroiliac joints and less in the puboischiadic joints. Relaxation began during the last month of gestation and persisted for some days after parturition. Castration during pregnancy did not interfere with the loosening of the pelvic ligaments.

2. Guinea Pig

Le Gallois (1812) was probably the first to describe the phenomenon of pubic relaxation in the guinea pig. He indicated that the separation took place during the latter part of pregnancy and was essential for birth of the very large young. Confirmation of this observation has been reported independently by various investigators (Duncan, 1854; Owen, 1866; Flower, 1885; Stirling, 1902; Bland-Sutton, 1911; Whitely, 1911). Pubic relaxation has also been observed at estrus (Pommerenke, 1933) and at the end of estrus (Brouha and Desclin, 1934). Further confirmation for relaxation at parturition has been obtained from the X-ray studies of Granzow (1930).

Histological studies of the pubic symphysis have been reported by Todd (1923), Kawata (1925) and Herrick (1928). Ruth (1936 and 1937) found that the symphysis of the male passes from an indifferent embryonic stage of hyaline cartilage into an osseochondral stage. Proliferation of cartilage and ossification take place at about the same rate ending in complete ossification. The symphysis of the female guinea pig passes from the indifferent to a chondrofibrous stage and then develops a fissure containing fibroblasts and connective tissue which ultimately give rise to a separation of the skeletal elements of the symphysis. In pregnancy the histological changes consist of a progressive vascularization of the symphyseal ligament, proliferation of connective tissue cells, lengthening of the ligament, and bone resorption toward the end of pregnancy. After parturition regressive changes comparable to cicatrization are seen in the symphyseal ligaments in addition to bone regeneration.

3. Mouse

Relaxation of the pubic symphysis of the pregnant mouse has been described by Gardner (1936) and Newton and Lits (1938). Gardner pointed out that the pubic symphysis of the male showed no change with age whereas a partial resorption leading to an increased mobility of the pubic bones occurred in the female. During pregnancy long interpubic ligaments were formed.

4. Other Species

In addition to the above forms, pelvic changes have been described in the dog (Frank, 1917), in the mole (Leche, 1880; Hisaw and Zilley, 1927), in the pregnant cow (Barlow, 1854), in the ewe (Franz, 1888), in the pregnant seal (Knox, 1839; Flower, 1885) and in the pocket gopher (Chapman, 1919; Hisaw, 1925).

5. Conclusion

It may be concluded that pelvic changes occur during pregnancy in the females of widely separated species of mammals. Thus far but a few have been carefully studied, i.e., the guinea pig and the mouse, and these will be considered in some detail. Of the remaining, only for the mole and pocket gopher (see Introduction) is any information available with regard to the controlling mechanism of pelvic adaptations.

III. Hormonal Factors in the Relaxation of the Symphysis Pubis

1. Guinea Pig

It has been agreed by most investigators that relaxation of the symphysis pubis of the guinea pig during pregnancy is under hormonal control. However, a disagreement arose with regard to the specific endocrine agents involved in this phenomenon. Hisaw (1926) first postulated that this reaction was under the control of a hormone which later was named relaxin. He presented evidence that this new factor was present in the blood serum of pregnant rabbits, guinea pigs, cats, dogs, and mares (Hisaw, 1926, 1927, 1929). He further showed that it was necessary to sensitize the castrated guinea pig first with estrogen in order to obtain pubic relaxation with relaxin-containing preparations. These studies were confirmed by Brouha and Simonnet (1928), Brouha and Desclin (1934), and Abramson *et al.* (1937). In the meantime, other investigators showed that pubic relaxation could be produced in the castrated guinea pig by estrogen alone (de Fremery *et al.* 1931; Courrier, 1931; Brouha, 1933; Dessau, 1935; Young and Emery, 1949) or by a combination of estrogen and progesterone (Fels, 1931; Möhle, 1933; Tapfer and Haslhofer, 1935; Haterius and Fugo, 1939; Courrier, 1941a, 1941b; Fugo, 1943). The ability of the sex steroids to produce pubic relaxation led many investigators to question the existence of such a hormone as relaxin.

Brouha and Desclin (1934) compared the relaxative action of two separate extracts of sow corpora lutea. The first was a water soluble preparation extracted for relaxative activity (Hisaw *et al.*, 1930), and the second was a lipoidal preparation extracted for progestational action. The water soluble extract (relaxin) produced relaxation in estrinized guinea pigs within 12 hours, while the lipide extract gave negative results. Estrogen alone required 20 to 25 days. Thus, these authors pointed out that on a time basis alone relaxation produced by relaxin differed from that produced by estrogen.

In spite of these experiments, the fact that relaxation could be produced by crystalline progesterone in the estrinized guinea pig caused many investigators to continue doubting the existence of relaxin. Courrier (1941a and b) reported that relaxation could be obtained with either estradiol or progesterone but when given together, the reaction occurred sooner and was more marked. These observations were confirmed by Hisaw *et al.* (1942, 1944), who showed that relaxation ordinarily occurred within 6 hours following the injection of relaxin, while a single dose of progesterone required 72 to 96 hours. Furthermore, relaxin was equally effective in castrated and castrated-hysterectomized guinea pigs, whereas progesterone was effective only in the castrated animal. Finally, as an explanation of these results, the authors showed that progesterone induced the formation of relaxin in the blood of a rabbit with an intact uterus but not in a hysterectomized rabbit. In 1943, however, Fugo reported relaxation of the pubic symphysis of the castrated and castrated-hysterectomized guinea pig with massive doses of estradiol dipropionate and progesterone and concluded the uterus was not essential for the relaxative action of progesterone.

Zarrow (1946, 1948) reinvestigated the question of steroid hormones and relaxin in the relaxation of the guinea pig symphysis. He was able to show that the three hormones, i.e., estradiol, progesterone, and relaxin, could separately produce relaxation but that the time requirement for each was significantly different. It is apparent from the data in Table I that estradiol will bring about public relaxation in both the castrated and castrated-hysterectomized animal and that the length of treatment required is essentially the same, i.e., 23.7 and 25.6 days, respectively. However, the addition of progesterone after 10 days of estradiol treatment shortened the time to 13.5 days in the castrated animal or 3.5 days after the start of progesterone injections. In the castrated-hysterectomized guinea pig the addition of progesterone had no effect on the time required for pubic relaxation. It is also apparent that estradiol produced relaxation in the same length of time regardless of the presence or absence of a uterus. However, a response to relaxin was noticed within 6 hours after the hormone was injected in either a castrated or castrated-hysterectomized animal previously treated with estradiol for 4 days. Also, it is important to point out that relaxin was found in the blood of the guinea pigs treated with estradiol and progesterone and having an intact uterus but not after estradiol treatment alone (Zarrow, 1948). From these results one may conclude that relaxation of the symphysis pubis of the guinea pig may be produced by either a prolonged treatment with estrogen or with relaxin following a short pretreatment with estrogen, and that the action of progesterone is an indirect one causing the formation of relaxin in the presence of an intact uterus.

Histological changes in the pubic symphysis lend added evidence in support of the above concept. Relaxation with estrogen results from a resorption of bone, proliferation of loose fibrous connective tissue, and an increase in mucoid, alkaline phosphatase, and water content of the symphyseal tissue (Talmage, 1947a, b, and c; Heringa and Van der

Meer, 1948). Relaxin produces a dissolution and splitting of the collagenous fibers into thin threads (Talmage, 1947b; Heringa and Van der Meer, 1948) and a breakdown of the glycoproteins (Perl and Catchpole, 1949). Talmage (1947b) found that the histological changes following treatment with progesterone were identical with those seen following an injection of relaxin. Thus it would appear that relaxation of the symphysis pubis of the guinea pig can be brought about by two quite different procedures. Relaxation produced by a prolonged treatment with estradiol results from growth and accumulation of tissue within the symphysis while relaxin modifies the existing connective tissue structures.

TABLE I

Time Required for Pubic Relaxation in the Castrated and Castrated-Hysterectomized Guinea Pig after Treatment with Estradiol, Progesterone and Relaxin[1]

Treatment—daily		Relaxin	Average relaxation time—days
Estradiol	Progesterone		
CASTRATED			
10 μg.	1 mg. from 11th day	....	13.5
10 μg.	....	....	23.7
1 μg.	....	2.G.P.U.[2]	4.0
CASTRATED, HYSTERECTOMIZED			
10 μg.	1 mg. from 11th day	....	23.7
10 μg.	....	....	25.6
1 μg.	....	2 G.P.U.[2]	4.0

[1] Data taken from Zarrow (1948).

[2] The relaxin was injected in one dose on the 4th day, after priming the animals with estrogen for 3 days.

The ability of desoxycorticosterone acetate to induce pubic relaxation in the estrogen primed guinea pig (Courrier, 1941a) is not surprising. Previous investigators have shown that both progesterone and desoxycorticosterone have similar action with regard to production of a progestational uterus (van Heuverswyn *et al.* 1939; Holweg, 1939; Robson, 1939), growth of mammary glands (Mixner and Turner, 1942), prolongation of life in the adrenalectomized animal (Emery and Schwabe, 1936; Gaunt and Hays, 1938; Schwabe and Emery, 1939), protection against cold (Zarrow, 1942) and withdrawal bleeding in monkeys (Hisaw, 1935; Speert, 1940). Using a massive priming dose of estrogen, Emery (1946) reported that progesterone and desoxycorticosterone were about equally active in their ability to produce relaxation while Zarrow found the ratio of activity of progesterone to desoxycorticosterone to be 1 to 10.

Relaxin has been found in the blood serum of castrated guinea pigs (Zarrow, 1948) and castrated rabbits (Hisaw *et al.*, 1944) following the injection of desoxycorticosterone.

Emery and Lawton (1947) have reported pubic relaxation in guinea pigs treated with estrogen and prostigmine and that hysterectomy did not affect the reaction. At present, information regarding the changes produced in the symphyseal tissue by prostigmine are insufficient to make a comparison with those produced by relaxin.

2. *Mouse*

The pubic symphysis of the male mouse remains cartilaginous, while in the female the cartilage is replaced by an interpubic ligament at sexual maturity (Gardner, 1936). During the first pregnancy the pubic bones are resorbed at their anterior end and the interpubic ligaments elongate. Pubic separation begins on the 13th to 15th days of pregnancy and reaches a maximum on the 19th day. The symphysis closes after parturition but never completely. A small permanent separation remains which is increased slightly with each succeeding pregnancy (Hall and Newton, 1946). Castration of the female mouse prior to puberty results in the retention of the male type of symphysis (Gardner, 1936) while castration and destruction of the fetuses prevents separation of the pubic bones (Newton and Lits, 1938). Furthermore, maintenance of pregnancy with progesterone in castrated mice results in a failure of the pubic bones to separate (Hall and Newton, 1946b). Hypophysectomy during pregnancy does not interfere with the separation of the pubic bones provided the placentae are not lost (Newton and Beck, 1939).

Treatment of normal and castrated male mice with large doses of estrogen for a long period of time led to pubic separation with the formation of an interpubic ligament 2 to 3 mm. long (Gardner, 1935; Burrows, 1935). Similar results were obtained with immature mice (Sutro, 1940) and castrated female mice (Gardner, 1936). Simultaneous treatment with estrogen and testosterone prevented pubic resorption (Gardner and Pfeiffer, 1938). Moreover, daily injections of testosterone propionate from the middle to the end of pregnancy inhibited the separation of the symphysis pubis (Gardner and Van Heuverswyn, 1940). Although it is obvious that estrogens can produce an interpubic ligament, the separation of the mouse symphysis is too rapid and extensive during pregnancy to be explained by the effects of estrogen alone. Evidence that a second factor may be present in pregnancy to account for the speed and the extent of the pubic separation has been obtained recently. Hall and Newton (1946a, 1947) treated castrated female mice with 1.6 to

25 μg. of estrone daily for 10 days and obtained a pubic separation of 0.20 mm. However, the injection of an extract of pregnant rabbit serum (an excellent source of relaxin) when the mice had been primed with estrone, produced a pubic separation of 4.33 mm. and the rate of separation approximated that seen in pregnancy. Treatment with nonpregnant rabbit serum gave negative results.

Contrary to the results obtained in the guinea pig, progesterone not only failed to induce pubic relaxation in the estrinized mouse (Hall and Newton, 1946b, 1947), but inhibited the effect of estrone on the separation of the pubic bones (Table II). Hall (1949) suggested that the inhibitory effect of progesterone is caused by an antagonism between progesterone and estrogen. Such antagonisms are discussed in detail by Courrier in this volume.

TABLE II

*Inhibition by Progesterone of the Relaxation of the Pubic Symphysis of the Mouse Treated with Esterone and Relaxin**

Treatment	Pubic separation mm.	Treatment	Pubic separation mm.
Estrone 1.5 μg. per day × 8	0.7	Esterone 1.5 μg. per day × 8 Progesterone 1 mg. per day × 8	0.3
Estrone 25 μg. per day × 14	1.1	Estrone 25 μg. per day × 14 Progesterone 1.5 mg. per day × 14	0.5
Estrone 1.5 μg. per day × 9 Relaxin 0.5 ml. on day 9	1.0	Estrone 1.5 μg. per day × 9 Relaxin 0.5 ml. on day 9 Progesterone 1 mg. per day × 9	0.2
Estrone 25 μg. per day × 9 Relaxin 0.5 ml. on day 9	0.9	Estrone 25 μg. per day × 9 Relaxin 0.5 ml. on day 9 Progesterone 1 mg. per day × 9	0 3

* Data taken from Hall (1949).

The histological changes noted during the last 5 to 6 days of pregnancy consist of a resorption of the pubic bones and lengthening of the interpubic ligament by formation of new cartilage and reversion of this cartilage to a primitive type of orientated, collagenous connective tissue. Twenty-five micrograms of estradiol daily for 10 days produced no change in the symphysis but the addition of relaxin produced a histological change similar to that described for pregnancy (Hall, 1947). This increase in collagenous fibers caused by relaxin is again unlike the situation in the guinea pig in which a dissolution of the collagenous fibers occurs (Talmage, 1947a).

IV. ASSAY OF RELAXIN

1. *Manual Palpation*

The assay for relaxin most commonly used is based on the relaxation of the pubic symphysis of the guinea pig (Hisaw, 1927), which can be determined by manual palpation. In the initial investigations all results were reported in a qualitative manner which served to detect the presence or absence of relaxin in the test fluids (Hisaw, 1929; Hisaw *et al.*, 1930). In 1930, Fevold, Hisaw, and Meyer defined a unit of relaxin as the minimum amount of hormone that caused a definite loosening of the pelvic ligaments within 10 to 12 hours after a single injection. The accuracy of this method was quite poor and the procedure laborious due to the necessity of making serial dilutions.

In an attempt to quantitate the assay, Abramowitz *et al.* (1942, 1944) established a standard dose response curve which had a typical sigmoid shape (Fig. 1). The increment of response is practically equal to the increment in dosage between $\frac{1}{8}$ and 2 g. equivalent of fresh corpora lutea. That this relationship is one of direct proportionality may be seen in Fig. 2 in which a semilogarithmic plot of the data yields a straight line relationship. On the basis of this curve one guinea pig unit of relaxin was defined as that amount of hormone which incuded an unmistakable relaxation of the symphysis pubis in two-thirds of a group of 12 castrated guinea pigs weighing between 350 and 800 g. All animals were pretreated with 0.83 μg. of estradiol daily for 4 days and the pubic symphysis palpated 6 hours following the injection of relaxin. This method of assay was found to be reasonably accurate and in addition gave quantitative results. The authors indicated, however, that by this procedure doses differing by 50% could be distinguished but not less. In a further modification it was found possible to prime the guinea pigs with 1 μg. of estradiol daily for 3 days in place of the previous 4 days and thus the assay was shortened by a day (Hisaw and Zarrow, 1948).

2. *X-Ray*

Hall (1948) has described an assay for relaxin using spayed mice and measuring the interpubic separation by X-ray photographs. For the

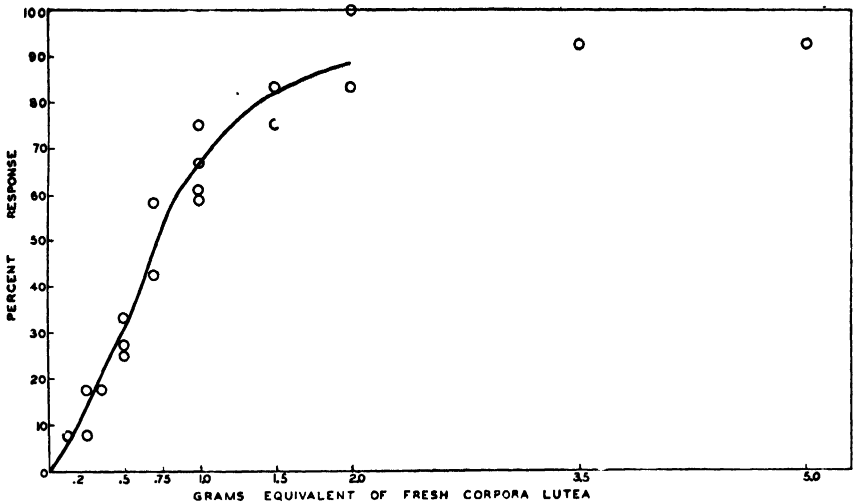

FIG. 1. Dose-response curve for relaxin. Percentage of guinea pigs showing relaxation of the symphysis pubis after a single injection of varying doses of relaxin. (Abramowitz *et al.*, 1944.)

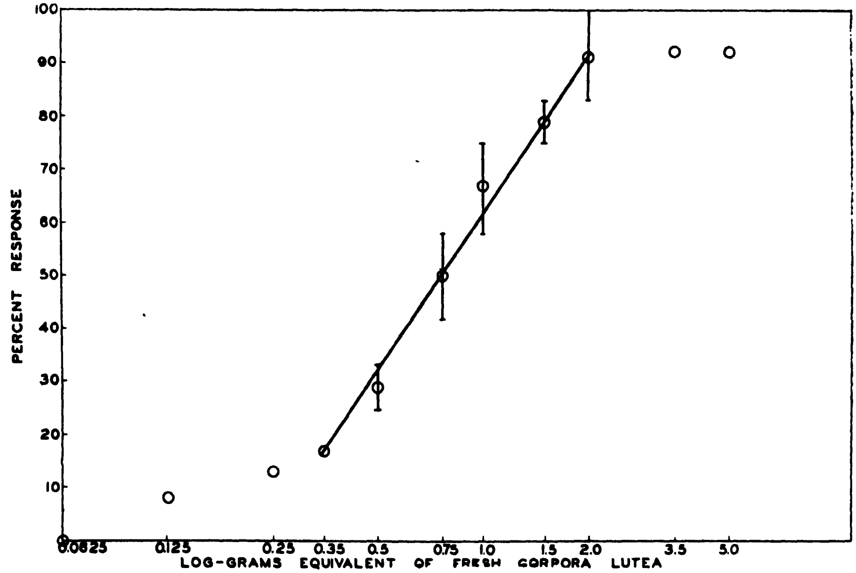

FIG. 2. Dose-response curve for relaxin. Percentage of guinea pigs showing pubic relaxation as a function of the logarithm of the dose. (Abramowitz *et al.*, 1944.)

best results the mice were primed with 1.5 μg. of estrone daily for 8 days and given a single injection of relaxin 24 hours after the final estrogen treatment. X-ray examinations of the pelvis were made 24 hours before and after the injection of the test dose of relaxin. Contrary to the results described in the guinea pig assay for relaxin (Abramowitz *et al.* 1944) it was found by Hall (1948) that continuous treatment with estrogen led to an increased sensitivity in the mouse. Salhanick and Zarrow (1949) found a great deal of variation in the amount of pubic separation in the mouse after treatment with relaxin.

The X-ray technique was applied to the guinea pig by Talmage and Hurst (1949). They found a high correlation between the palpation and X-ray methods. The variation however was so great as to require fifty animals at each of three dose levels to reduce the error of the assay to 20%.

3. Seasonal Variations

The response of castrated guinea pigs to one guinea pig unit (G.P.U.) of relaxin was determined periodically for one year in order to ascertain whether changes in the population of the guinea pig colony and seasonal variations influenced the assay. A standard relaxin powder (J-46) originally assayed at 30 G.P. units per mg. dry weight was used throughout the experiment. The relaxin was injected subcutaneously into groups of 12 guinea pigs previously primed with 1 μg. of estradiol daily for 3 days. It is apparent from an examination of the data (Fig. 3) that no seasonal variations in the assay occurred. The response varied from 50 to 80% with an overall average of 67 ± 15% for the year's data. This checks extremely well with the initial assay of the extract, which gave a 67% response in 30 animals and further indicates that changes in population of the colony also had no effect on the assay.

V. THE CHEMISTRY OF RELAXIN

1. Corpora Lutea

Relaxin has been extracted from the corpora lutea of sows by several different methods. In the initial experiments of Hisaw *et al.* (1930) and Fevold *et al.* (1930a, b, and c) ground luteal tissue was extracted with 2% HCl in 95% ethanol. On neutralization and evaporation of the filtrate to semi-dryness a starting material was obtained from which the hormone was further concentrated by a procedure that involved primarily repeated fractionations with ethanol and ether. Abramowitz *et al.* (1944) and Albert *et al.* (1946) extracted dried, defatted, luteal tissue with aqueous 2% HCl and concentrated the hormone by fractional precipitation with

$(NH_4)_2SO_4$ and ethanol. These procedures yielded preparations that contained 20 to 30 G.P. units per mg. dry weight.

The chief chemical characteristics of relaxin, as judged by these extracts (Fevold *et al.*, 1930a, b, c), were its isoelectric point of 5.4 to 5.5, stability in acid solutions, and its insolubility in the common nonpolar solvents. Alkalis, oxidizing agents and proteolytic enzymes like trypsin and pepsin destroyed the activity of the hormone. The preparations were amphoteric in nature and had a nitrogen content of 11%. It was suggested that relaxin may have a peptide-like structure though it appeared to be somewhat different from typical peptides and polypeptides. Relaxin, due to its chemical properties, was easily separated from the progestational hormone of the corpus luteum (Fevold *et al.*, 1932).

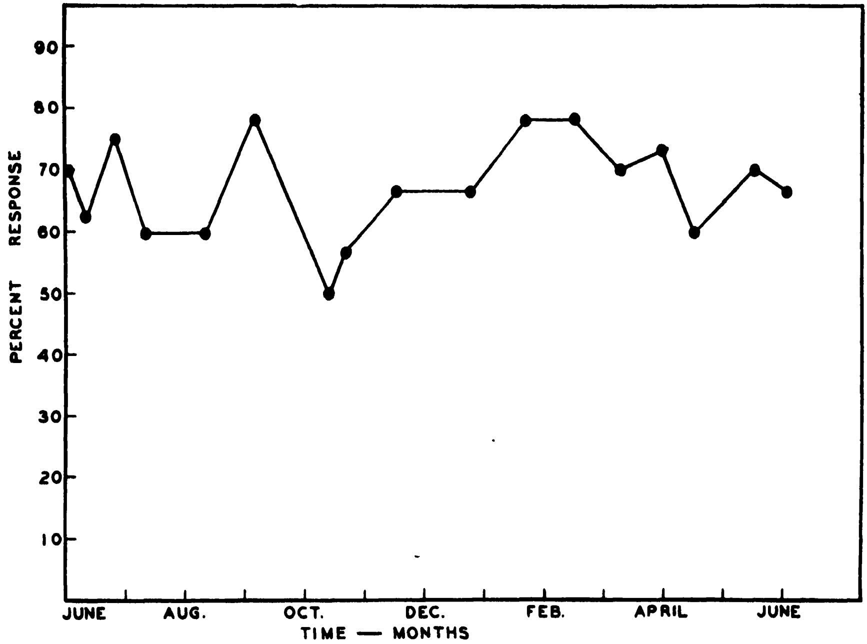

FIG. 3. Response of castrated guinea pigs to one unit of a standard relaxin powder (J-46) tested every month for one year. Each point represents the percentage of guinea pigs showing pubic relaxation plotted against time of year.

2. Whole Ovaries

Albert *et al.* (1947) have reported a simplified procedure for the extraction of relaxin from fresh, whole ovaries of the sow. The ovaries are finely ground and extracted with four volumes of 2% HCl, filtered, and the hormone adsorbed on bentonite. The relaxin is removed from the bentonite with 6% pyridine and further purified by isoelectric precipitation. With these preparations, activity could be demonstrated upon the injection of single doses of 2 to 10 μg., corresponding to a potency of 100 to 500 G.P. units per milligram of dry powder. However, this method of concentrating the hormone resulted in a loss of approximately two-thirds of the activity contained in the starting material.

The discovery that fresh whole ovaries of the sow are a better source of relaxin than dried corpora lutea has made it less difficult to prepare highly potent preparations of relaxin. While the luteal tissue contained 1 to 4 G.P. units per g., the whole ovaries of nonpregnant sows had 3 to 10 units per g. of fresh tissue and those of pregnant sows 3000 to 9600 units (Albert *et al.*, 1947). Thus it is possible, by using ovaries of pregnant sows, to start with a material that contains as high as 10 G.P. units per mg. fresh weight.

3. Blood Serum

It was originally shown that relaxin was present in the blood of various animals during pregnancy, especially the rabbit, and it appeared to be combined with the pseudoglobulins (Hisaw, 1926, 1929). In 1930, Fevold *et al.* (1930a, b, c) indicated that the hormone could be obtained from the serum by evaporation to semi-dryness and extraction with saline or acidified 95% ethanol. Further work with sodium sulfate as a precipitating agent showed the relaxin activity to be present in the globulin fraction and specifically in the pseudoglobulins.

Abramson *et al.* (1937) fractionated serum with ammonium sulfate and found relaxin present in all three fractions, i.e., globulins, pseudoglobulins, and albumin. They concluded that an acid alcohol extract gave the most potent preparation. Albert and Money (1946) found that 30 to 50% cent of the hormone was lost when the above methods were used and consequently they were not suitable for quantitative experiments. They added one volume of cold ethanol to one volume of serum, dissolved the precipitate in saline and added another volume of cold ethanol. The two supernatants were combined, added to 4 volumes of acetone and the precipitate suspended in ⅕ volume of saline. This preparation showed a concentration of five to tenfold over the serum and gave complete recovery.

4. Urine

Albert and Money (1946) devised a method for extracting relaxin from urine. The urine was dialyzed against running tap water and distilled water, then reduced to $\frac{1}{2}$ its original volume by evaporation in a current of warm air, and poured into 4 volumes of cold acetone. Precipitation was facilitated by adding a few drops of saturated sodium chloride, the precipitate collected by centrifugation and suspended in saline so that 1 ml. was equivalent to 100 ml. of urine. This procedure gave a 10% loss in relaxin activity, probably occurring during dialysis.

VI. THE PHARMACOLOGY OF RELAXIN

1. *Time Required for Onset of Pubic Relaxation in the Guinea Pig after Treatment with Relaxin*

The interval elapsing between injection and appearance of a response varies from seconds to days for the different hormones. The biological effects of epinephrine, insulin, pituitrin, or intermedin become apparent in an extremely short time whereas in general the delay after injection of the sex hormones is much longer for most reactions (Zondek and Sulman, 1945). The action of relaxin, as noted by its effect on the symphysis pubis of the guinea pig, lies somewhere between these two extremes.

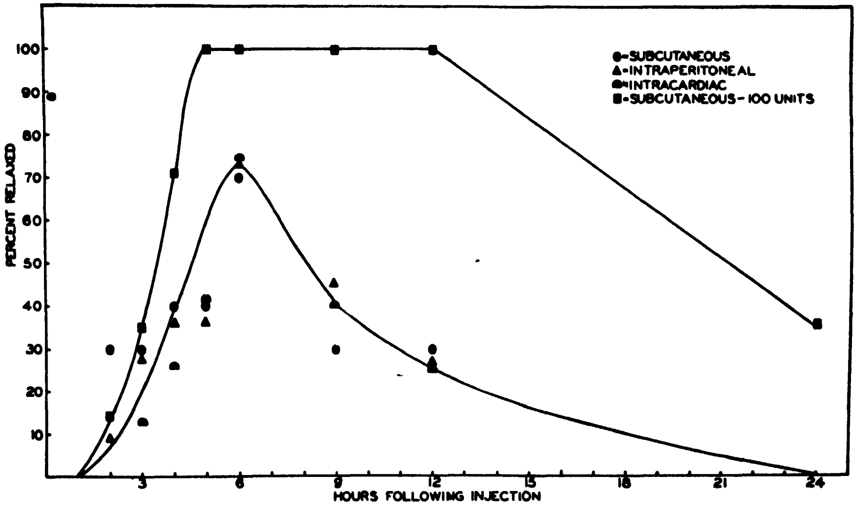

FIG. 4. Time required for relaxation of the symphysis pubis of castrated guinea pigs primed with estradiol and given a single injection of relaxin. The per cent response is plotted against the time in hours after the injection of relaxin. (Zarrow and Money, 1948.)

● Subcutaneous—1 G.P. unit of relaxin
▼ Intraperitoneal—1 G.P. unit of relaxin
◓ Intracardiac—1 G.P. unit of relaxin
■ Subcutaneous—100 G.P. units of relaxin

Hisaw (1927) based his observations on the response at the end of 12 hours while Abramowitz *et al.* (1944) found that a maximum relaxation of the symphysis pubis in a group of guinea pigs produced by a subcutaneous injection of 1 G.P. unit was obtained in 6 hours.

Zarrow and Money (1948) made the further observation that a maximum response was obtained in 6 hours after the injection of 1 G.P. unit of relaxin by either the subcutaneous, intraperitoneal or intracardiac route. A small percentage of the animals showed relaxation as early as 2 hours. Also, the time response curve was the same for the various routes of injection. By 9 hours after treatment the response began to disappear and by 24 hours the symphyses were no longer relaxed. Though the subcutaneous injection of 100 G.P. units of relaxin did not decrease the time required for the first appearance of pubic relaxation, the time for maximal response was shortened to 4 hours and the peak response was maintained for 7 hours (Fig. 4).

2. Rate of Disappearance of Relaxin from the Blood Stream

Marder and Money (1944) have shown that the relaxin content of the blood of pregnant rabbits rapidly decreases following parturition. In 72 hours the blood level falls from 10 G.P. units per ml. of serum to less than 1 unit. After a single intravenous injection of relaxin, approximately 50% is lost from the blood in 1 hour and by 24 hours the hormone is no longer detectable. The subcutaneous injection of relaxin gave the same type of curve (Fig. 5).

It was also shown that relaxin is absorbed very rapidly into the blood when injected subcutaneously. A maximal concentration of relaxin was obtained in the blood 5 minutes after a subcutaneous injection (Zarrow and Money, 1938). Determinations of the amount of relaxin in the urine indicated that from 1 to 4% of the injected hormone had been excreted in an active form. It would appear that this material is very rapidly broken down in the body or for the most part excreted as a degraded and inactive substance.

3. Toxicity

Relaxin has not been obtained in a chemically pure state and until that is accomplished its toxicity can not be determined with a high degree of validity. However, Zarrow and Money (1946) have presented suggestive data for impure preparations. They obtained an LD.50 in mice after a single subcutaneous injection of 40,000 G.P. units of relaxin per kg. body weight. When the mice received the preparation in three divided doses in one day an LD.50 of 30,000 G.P. units per kg. body weight was observed. These values were obtained with a relaxin preparation having a potency of 30 G.P. units per mg. dry weight. A second preparation with a potency of 250 G.P. units per mg. dry weight produced no adverse effects when given, as single subcutaneous or intravenous injection, at a dose level of 80,000 G.P. units per kg. body weight.

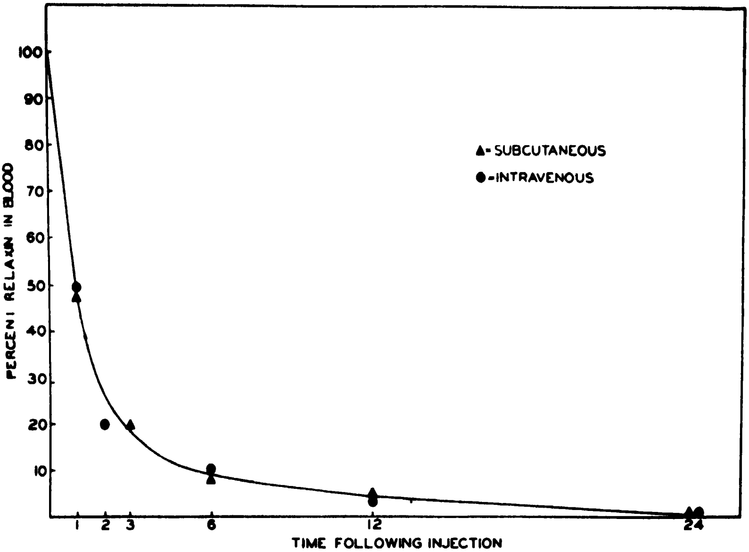

FIG. 5. Rate of disappearance of relaxin from the blood following intravenous and subcutaneous injections of the hormone. The concentration of relaxin is expressed in percentage by considering the amount of hormone in the blood serum five minutes after injection as 100%. (Zarrow and Money, 1948.)
▲ Subcutaneous
● Intravenous

In the rabbit, however, an occasional fatality has resulted from intravenous injections of 5000 to 10,000 G.P. units of relaxin (Zarrow, 1949b).

4. Antibody Formation

Several attempts have been made to establish whether or not relaxin had antigenic properties and so far all have given negative results. Zarrow and Money (1948) failed to develop antibodies to relaxin in the rabbit after a series of treatments. This is in agreement with the fact that castrated guinea pigs can be used repeatedly for more than a year in the assay of relaxin without loss of sensitivity to the hormone or the occurrence of anaphylactic reactions. These observations indicate that relaxin is either weak or lacking in antigenic potentialities.

VII. SOURCES OF RELAXIN

The ovary, uterus, and placenta have been suggested as possible sites of relaxin formation (Hisaw, 1926, 1929; Herrick, 1928; Hisaw *et al.*, 1944; Zarrow, 1946). Not only has it been possible to extract relaxin from these three organs but there is indication that they may serve as sources of the hormone in the normal physiology of the animal. Castration of the pregnant guinea pig at mid-term (Herrick, 1928; Courrier *et al.*, 1938) and hypophysectomy on the 40th day (Pencharz and Lyons, 1934) failed to interfere with normal pubic relaxation. Thus, the presence of a normal placenta appears able to ensure a sufficiency of relaxin during pregnancy in the absence of both the pituitary and ovary.

A normal concentration of 10 G.P. units of relaxin per ml. of blood serum is maintained in pregnant rabbits when they are castrated at mid-term and abortion is prevented by giving progesterone. In one rabbit given such treatment a fall in relaxin content of the serum to 5 G.P. units per ml. was noted and at post mortem the animal was observed to have only two viable fetuses (Zarrow, 1947b), possibly indicating a partial relationship between the relaxin concentration of the blood and the amount of placental tissue. This evidence, plus the fact that the placenta contains more relaxin than the uterus (Table III) and the precipitous loss of relaxin from the blood at parturition, points to the placenta as the chief source of relaxin in the pregnant rabbit.

The observations of Hisaw *et al.* (1941) indicate that the uterus of the rabbit is also capable of producing relaxin. It was found that the blood of rabbits, castrated during estrus and given progesterone for 9 days, contained 1 G.P. unit of relaxin per 5 ml. of serum. This is similar to the concentration found in the blood of the rabbits during pseudopregnancy and on the 9th day of pregnancy. Failure to find relaxin in the blood of male rabbits and castrated, hysterectomized rabbits treated in a similar manner indicated strongly that the uterus was the source of relaxin in these experiments. Similar results have been obtained for the guinea pig.

It is of interest that the formation of relaxin in the mouse is apparently different from that observed in guinea pigs and rabbits. Like the rabbit, the presence of a placenta seems to be necessary for the normal relaxation of the pubic symphysis of the pregnant mouse. Hypophysectomized pregnant mice show normal pubic relaxation if the placentae are retained, while loss of the placentae results in a failure of the pubic bones to separate (Newton and Beck, 1939). Hall and Newton (1947) suggest that in the mouse the placenta acts by stimulating the production of relaxin in the ovary. It may be that the ovary is the principal source of relaxin in the pregnant mouse and that its production is under the control of the luteotropic hormone of the placenta (Astwood and Greep, 1938). At least it appears that the action of progesterone on the uterus is not an important factor and in this respect mice differ from rabbits and guinea pigs.

TABLE III

Relaxin Content of Various Tissues in Different Species

Species	Tissue	Stage of reproductive cycle or type of treatment	Relaxin content G.P.U.	References
Sow	Ovary	Immature	< 1/g.	Hisaw and Zarrow (1948)
Sow	Ovary	Follicular	< 1/g.	Hisaw and Zarrow (1948)
Sow	Ovary	Luteal	2.5–5/g.	Hisaw and Zarrow (1948)
Sow	Ovary	Nonpregnant	3–10/g.	Albert *et al.* (1947)
Sow	Ovary	Pregnant	10,000–15,000/g.*	Hisaw and Zarrow (1948)
Sow	Ovary	Pregnant	3000–96000/g.	Albert *et al.* (1947)
Sow	Placenta	Pregnant	0.5–2.5/g.	Hisaw and Zarrow (1948b)
Sow	Uterus	Pregnant	< 1/g.	Hisaw and Zarrow (1948b)
Sow	Blood serum	Pregnant	2/ml.	Hisaw and Zarrow (1948b)
Guinea pig	Blood serum	Nonpregnant	Negative	Zarrow (1947a)
Guinea pig	Blood serum	Pregnant	0.5/ml.	Zarrow (1947a)
Guinea pig	Uterus	Estradiol progesterone	10/g.	Zarrow (1948)
Rabbit	Blood serum	Nonpregnant	Negative	Marder and Money (1944)
Rabbit	Blood serum	Pregnant	10/ml.	Marder and Money (1944)
Rabbit	Blood serum	Pseudopregnant	0.2/ml.	Hisaw *et al.* (1944)
Rabbit	Blood serum	Progesterone	0.2/ml.	Hisaw *et al.* (1944)
Rabbit	Uterus	Pregnant	10/g.	Zarrow (1949b)
Rabbit	Placenta	Pregnant	50–75/g	Zarrow (1949b)
Rabbit	Ovary	Pregnant	25–30/g	Zarrow (1949b)
Human	Blood serum	Pregnant (First 6 mos.)	Present	Abramson *et al.* (1937)
Human	Blood serum	Pregnant (First 6 mos.)	Present	Pomerenke (1934)
Dog	Blood serum	Pregnant	Present	Hisaw (1929)
Cat	Blood serum	Pregnant	Present	Hisaw (1929)
Mare	Blood serum	Pregnant	Present	Hisaw (1929)
Chicken	Ovary	....	10/g.	Zarrow *et al.* (1947)

* These data were obtained by assaying crude extracts of single ovaries of pregnant sows without any attempt at purification.

There even may be some question as to whether progesterone takes a direct part in eliciting the formation of relaxin in pregnant mice. The pubic symphyses remain closed in castrated mice in which gestation is maintained by injecting progesterone. As a matter of fact, there is evidence that progesterone actually has an inhibitory effect on pelvic relaxation in mice (Hall, 1949 and see Section III, 2 above).

The ovary in the domestic sow seems to be the principal source of relaxin (Hisaw and Zarrow, 1948). The hormone is extremely plentiful in the ovaries during pregnancy and reaches its highest concentration by the time the fetuses are 5 or 6 inches in length (Table III). The uterus and placenta contain very little. During the estrus cycle, relaxin is present in the ovaries only in the luteal phase and the amount is small in comparison with that of advanced pregnancy.

VIII. ACTION OF RELAXIN OTHER THAN ON THE PELVIC LIGAMENTS

1. *Mammary Gland*

Hamolsky and Sparrow (1945) have reported a synergistic action of relaxin on the effects of estrogen and progesterone on the development of the mammary glands in rats. Treatment with estradiol, progesterone and relaxin caused growth and lobulation of the mammary glands in immature castrated rats comparable to that seen in pregnancy whereas estradiol and progesterone alone gave only slight growth. Estradiol and relaxin or progesterone and relaxin gave negative results. Also, negative results were obtained in the hypophysectomized rat after treatment with all three hormones. Confirmation of these results have been obtained by Smith (1950).

2. *Vaginal Mucosa*

Dewar *et al.* (1946) have reported a potentiation of the vaginal response to estrone by relaxin. Using the "cross over" test in castrated mice and vaginal cornification as an index of activity, these investigators found that the effect of estrone was increased from 7.5 to 50-fold by the addition of 2.5 G.P. units of relaxin extracted from pregnant rabbit serum.

3. *Antidiuretic Action*

Preparations containing relaxin have been shown to have an antidiuretic action in rabbits (Zarrow, 1949a). Female rabbits injected with 250 to 1000 G.P. units of relaxin three times daily for 3 days showed a 60 to 80% drop in urine output (Fig. 6C and D). In one instance continued injection of 570 G.P. units of relaxin three times daily produced an immediate antidiuresis and death after 12 days of treatment. A similar treatment with 250 G.P. units failed to maintain an initial antidiuretic state and a normal urine output returned within 28 days and remained normal until termination of the experiment at 40 days (Zarrow, 1947b). A number of preparations that contained relaxin were tested for antidiuretic action. It was found that those prepared from the ovaries of pregnant sows and nonselected ovaries caused urine retention while a preparation from beef heart or an equivalent volume of

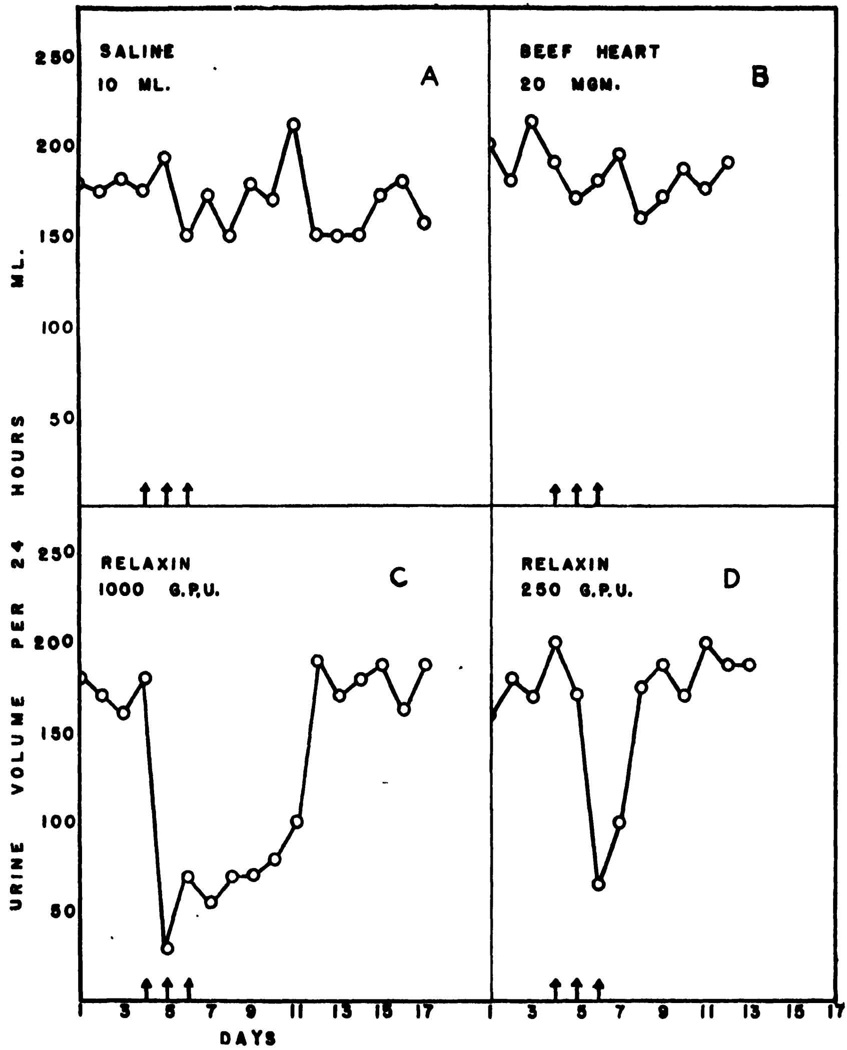

FIG. 6. Antidiuretic action of preparations containing relaxin. (Zarrow, 1949a.) A. Lack of effect on urine output after treatment with 10 ml. of physiological saline injected 3 times daily for 3 days. Days of treatment indicated by arrows. B. Lack of effect of 20 mg. equivalent of beef heart extract injected 3 times daily for 3 days. C. Marked antidiuretic response obtained with 1000 G.P. units of relaxin (J-46) injected 3 times daily for 3 days. D. Antidiuretic response obtained with 250 G.P. units of relaxin (J-46) injected 3 times daily for 3 days.

saline had no effect on fluid excretion. Probably the most significant observations were those for rabbit serum. A preparation of relaxin obtained from the blood serum of pregnant females gave a definite antidiuretic reaction while a similar preparation from the blood serum of males, in which there was no relaxin, gave a negative response.

It was also found that the minimum effective dose in terms of relaxin for an antidiuretic action was about 250 G.P. units (as defined by Abramowitz *et al.*, 1944) and that the antidiuretic activity of a preparation paralleled its relaxin content. In addition it should be mentioned that dialysis studies indicated that the antidiuretic factor is not identical with posterior pituitary hormone.

4. Cellular Components of the Blood

In conjunction with the investigation on the antidiuretic action of relaxin, hematocrit studies indicated that one of the accompanying effects was a definite shift in water balance. This led to a study (which is still in progress) of the changes that occur in the cellular components of the blood during pregnancy and after the injection of relaxin. Preliminary results indicate that the rabbit develops a pregnancy anemia similar to that recently described for the rat (Newcomer, 1947). These changes include a drop in hematocrit and erythrocytes and an increase in reticulocytes. These same conditions have been observed in normal and castrated female rabbits receiving relaxin (Zarrow and Zarrow, 1949). Injections of 250 to 500 G.P. units thrice daily in a small number of rabbits have consistently caused an erythrocyte and hematocrit drop of approximately 33% and a rise in the reticulocytes, while cessation of treatment was followed by a return to normal values.

IX. General Discussion

If the conditions under which relaxin attains its greatest concentration in the body are taken as an indication of its basic function, then one must conclude that it is a hormone of pregnancy. The formation of relaxin by the female reproductive tract in response to progesterone, relaxation of pelvic ligaments, retention of body fluid and associated changes in the blood of rabbits, and effects on mammary development in rats all support this view. However, such reasoning may lead to erroneous conclusions. Relaxin may not be a hormone peculiar to the female and its formation may not be evoked only by luteal action. The effects seen during gestation could be physiological adaptations of a hormone that has a more general function. Instances of the acquirement of new functions by existing hormones are well known to students of reproduction and especially to those interested in the evolution of viviparity among the vertebrates.

It is important in this respect to point out that pelvic relaxation is not common to all mammals and in those in which it occurs the process involved may not be the same in all instances. In species in which the symphysis pubis responds to estrogen as a secondary sexual character, separation of the pelvic bones may be accomplished solely by bone resorption and growth of connective tissue. When relaxin takes part, as in the rabbit, guinea pig, and mouse, the reaction may involve only the ilio-sacral union (rabbit) or primarily the symphysis pubis (mouse) or both (guinea pig). It is also of interest that relaxin is known to be present in the blood of a number of species which apparently show little or no pelvic relaxation at parturition.

If pelvic relaxation is an adaptive response to relaxin then the question of its basic function remains unanswered. Probably an approach to a suitable answer is indicated by the nature of its action on the pubic symphysis of the guinea pig. In this reaction, the symphyseal tissue must first be acted upon by estrogen before it can respond to relaxin. The reason for this is not known but it may mean that the tissue of the symphysis must be undergoing growth. The first effect of relaxin is a dilation of the blood vessels in the connective tissue of the symphysis followed by the imbibition of fluid, modification of the ground substance, and dissolution and splitting of the collagenous fibers. Whether connective tissue structures in other parts of the body are similarly affected is not known. That a basic physiological process is common to pelvic relaxation, fluid retention, and changes in the blood resulting in "pregnancy anemia" during normal gestation is not clear; yet it is of considerable interest that these conditions can be produced in the non-pregnant animal by administering preparations of relaxin.

The known sources of relaxin also furnish rather interesting comparative data. In the pregnant sow, the ovaries seem to be chiefly responsible for the secretion of relaxin whereas in the rabbit and guinea pig the uterus and placentae are primarily responsible. These organs have no obvious morphological structure in common that can be singled out as a possible source for the hormone. Even in the sow's ovaries, the concentration of relaxin appears as great in the stroma as the corpora lutea, and it is absent in the follicular liquor. The only correlation that seems possible is between the formation of relaxin in these organs and the presence of progesterone.

The dependence of relaxin on the steroid hormones for its formation and action and the apparent absence of a specialized tissue for its secretion present a number of questions. Probably the most important is whether relaxin is a hormone of pregnancy and thus limited to the female or whether it may also have a more general function and be present in the male. The fact that desoxycorticosterone can cause the secretion of relaxin is suggestive as it indicates that the adrenal cortical steroids, common to both male and female, possess this potentiality. That relaxin has not been demonstrated in the male may be due to a low concentration in the blood and the inadequacy of our test methods. The high concentration in the blood and in tissues of the reproductive tract of pregnant females may be, as has been mentioned, the result of an adaptive specialization of these structures for the elaboration of relaxin, and thus they respond more readily to progesterone than other regions of the body that may share this potentiality to some degree, but in which a much stronger stimulus is required to evoke an equivalent response. Differential responses of this nature are known for certain steroid hormones, for example, the estrogens, and if such be true for the formation of relaxin then one may expect to find the hormone in the male and castrated-hysterectomized female under normal and experimental situations in which progesterone and probably adrenal cortical steroids are present in large amounts. Regardless of the validity of such speculation, it has been shown that progesterone does produce several reactions indirectly by causing the formation of relaxin, which is a sufficient reason for considering the possibility that other steroid hormones may in some instances produce effects by way of relaxin or similar substances.

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Interactions between Estrogens and Progesterone*

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I. Introduction

The two female sex hormones—the estrogenic and the progestative substances—do not act independently. They are bound by functional relations which constitute one of the many aspects of the vast problem of endocrine balance.

The study of these relations is important to biology and to medicine, but they are complex. The follicular estrogenic hormone—still often called folliculin—and the progesterone of the corpus luteum can assist one another in certain cases, but in other instances they exert interfering or even opposing action. Such synergism or antagonism takes place in close relationship to the receptors on which the hormones act. Further, it must be made clear that progesterone plays a part in the metabolism of estrogens, and that estrogen itself affects the activity of the ovarian corpus luteum.

It is only reasonable to attempt to apply our knowledge of these relationships, so important in normal physiology, to medicine. Perhaps in this way the genital diseases of the female can be better attacked. Certain specialists have even had the idea of utilizing the antagonism between progesterone and estrogen in their researches on tumors.

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The terms antagonism and synergism have been criticized (Seegar Jones and Astwood, 1942) in regard to mucification of the vagina, but discussions of nomenclature have only limited importance; facts are worth more than words. The facts show, for instance, that estrogens, under certain conditions, neutralize the normal action of progesterone on the uterine mucosa of the rabbit, and that progesterone neutralizes the normal action of estrogens on the sexual skin of the monkey, on the myometrium of the rabbit and on the secretion of the glands of the cervix of the uterus in women. Is this not to be considered an antagonism, a hormonal conflict, in the course of which certain structural changes (vagina) are produced? The name given to such indubitable phenomena is unimportant.

II. The First Experiments

After the preparation of the follicular hormone (estrogen) and before that of progesterone, some eminent endocrinologists of different countries defended the doctrine of a single ovarian hormone. I was one of those who reacted against this tendency, beginning in March 1926 (Courrier and Potvin, 1926). We know now that the two ovarian hormones, isolated in the pure state, each have a separate influence on the genital tract. During the ovarian cycle they act one after another on the genital organs, but they can also coexist in the same organism. Furthermore, experiments show that estrogen and progesterone interact when injected into the castrated female either successively or simultaneously.

In 1926 M. G. Smith with rats, and Parkes and Bellerby with mice, noticed that an extract of follicular fluid was able to interrupt gestation. They showed also that the injected follicular hormone reacted with difficulty on the vaginal epithelium when the ovary contained corpora lutea. This is the first reference to *antagonism:* the follicular hormone was thought to cause abortion by opposing the corpus luteum of pregnancy. But this hypothesis of hormonal antagonism was rapidly abandoned by Parkes (1930). I have myself criticized it following experiments (referred to later) on the differential thresholds of certain uterine responses in the rabbit. In addition, in 1931, Levin *et al.* denied that crystalline estrone has a tendency to cause abortion in the rat. They thought that the abortion obtained previously with follicular extracts (follicular abortion) was due to toxic impurities. Following Smith, Parkes, and Bellerby, follicular abortion was obtained by Courrier (1928) in the rabbit, by Zondek and Aschheim (1928) and Gostimirovic (1930) in the mouse, and by Kelly (1931) in the guinea pig.

In 1928, Loeb and Kountz stated that the follicular hormone tended to prevent the predecidual proliferation of the uterus, without studying at the same time its action on traumatic deciduoma. But what contributed at this time to the rejection of the idea of an antagonism between estrogen and progesterone was the fact that the relation between these substances was rather one of *synergism.*

The idea of synergism started in Hisaw's laboratory; preliminary action of the follicular hormone was found to be necessary in order for an extract of corpus luteum to open the pubic symphysis of the guinea pig. In March 1928, his pupil Weichert produced in the castrated rat and guinea pig the progestative state of the uterus (deciduoma of Loeb) by means of a corpus luteum extract, when, and only when, the uterus of the castrated animal had previously been exposed to the action of the follicular hormone. The necessity of this previous exposure has been confirmed by Brouha and Simonnet (1928), and later by other authors.

In April 1930, three publications appeared simultaneously: one by Hisaw and Leonard, another by W. Allen, and still another by Courrier.

Hisaw and Leonard, operating on the rabbit, were able to show that their corpus luteum extract acted on the endometrium only when it was previously sensitized by the follicular hormone. They assumed that the follicular hormone is equally necessary *during* the luteal action in order to maintain its effectiveness, because they had shown that if the treatment were continued for a certain length of time the uterus returned to rest in spite of continued injections of the luteal extracts.[1] They therefore injected extracts of the follicle and of the corpus luteum at the same time, but they observed that the corpus luteum extract no longer acted on the uterine mucosa; in trying to demonstrate synergism they actually had obtained antagonism.

In the same journal, W. Allen confirmed the necessity of a preliminary action of estrogen on the uterus of the rabbit in order that the progestin (Corner and Allen, 1929) could react. In certain experiments Allen injected estrogen and progestin simultaneously, and showed a normal action of progestin on the endometrium; he found no antagonism with the quantities then used.

At the same time that the American authors published their results, I published mine, first with guinea pigs and then with rabbits (Courrier, 1930a, b, c). At that time, extracts of corpus luteum were rare, their activity was irregular, and they contained some estrogen. I administered the follicular hormone in variable doses to animals having ovarian corpora lutea of the same age. For this purpose it was sufficient to choose guinea pigs which had just had a litter, since ovulation occurs regularly about twenty hours after parturition. When the uterine mucosa was traumatized 5 days after parturition, 6 days later a fine deciduoma of Loeb developed at the site of the lesion. If, however, these females in the luteal phase were injected with follicular extract, the deciduoma was reduced in size proportionally to the quantity of estrogen injected. If the estrogen were injected in a strong dose at the beginning of the luteal phase, the decidual reaction was totally absent. The follicular hormone, therefore, exercises a "veto" on the corpus luteum action. This same veto was readily found also in the rabbit; folliculin injected into a rabbit that has active corpora lutea prevents in some doses the formation of the characteristic progestational condition of the endometrium, which, in France, is known as the uterine 'lace' of Bouin and Ancel.

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[1] As Leonard, Hisaw, and Fevold (1932) mention, Courrier and Kehl in January (1930a) had noted that a pituitary extract given at the end of a pseudogestation in the rabbit caused the appearance of new corpora lutea, but the progestational changes of the uterus continued to regress.

These results are the reason for the title of my publication of April 1930, "The antagonism between the ovarian hormones." But synergism was not forgotten, for, in trying to obtain a deciduoma in the sterile horn of a rabbit in unilateral gestation, folliculin was injected in small doses before wounding the endometrium (Courrier and Kehl, 1930b).

Following these preliminary papers, I wrote the following sentence in a report to the Plenary Meeting of the Société de Biologie (Courrier 1931); "The intensively folliculinized uterus becomes incapable of reacting to progestin, while a weak dose of folliculin is indispensable for this reaction. The normal physiological processes take place without doubt within quantitatively narrow hormonal limits."

III. Antagonism in the Uterus

Hormonal antagonism takes place within the confines of the receptors. This was demonstrated (Courrier and Poumeau-Delille, 1943) in the following manner: a castrated rabbit is first given a subcutaneous injection of 2.5 μg. of estradiol daily for 6 days, to sensitize the uterine mucosa. After 24 hours' rest, the rabbit is injected in the same way with 0.5 mg. progesterone daily for 4 days to produce a progestational endometrium; but in addition a closed cavity is formed in each of the uterine horns, which insulates a fragment of horn between two ligatures 2 cm. apart. An oily solution of estradiol (20 μg.) is introduced in one cavity, and in the other the same quantity of oil without estradiol. The appearance of the progestational reaction was found in all parts of the uterus, except the part where the estradiol was placed. The antagonism is therefore manifested locally in the part which has been in direct contact with the estrogen.

This antagonism is not as crude a process as might have been thought. On the contrary, it is a very sensitive phenomenon which can begin and disappear rapidly. For example, a temporary suspension of the action of the corpus luteum on the endometrium was obtained in the following way (Courrier and Kehl, 1932): a rabbit was injected with folliculin for the first 4 days of the luteal phase; with certain doses of estrogen the progestative 'lace' of the uterus was absent on the sixth day, but became well developed on the twelfth day, i.e., the corpus luteum re-exerts its influence as soon as the effect of the estrogen has ceased.[2] Leonard *et al.* (1932) found a similar temporary suspension in their researches (rabbit RA 147).

It is possible, by means of this antagonism, to bring about a dissociation between the different uterine manifestations of the luteal phase. Thus in the rabbit, with small quantities of folliculin, we have been able to suppress the implantation of eggs without opposing formation of the endometrial 'lace.' Such a dissociation was obtained in 1928; the injected folliculin extract prevented implantation without preventing the progestative preparation of the endometrium, but at that time the experimental results were not understood. It is certain that the different manifestations of the luteal phase are not all attacked to the same degree by the same dose of folliculin; each has a threshold of resistance which appears to depend on the characteristic sensitivity towards progesterone. The existence of differential thresholds in the uterine reactions of the luteal phase was reported with Kehl (1933a). In our laboratory, A. Brouha (1934), who showed that folliculin also antagonizes the deciduoma of Loeb in the rabbit, studied these different thresholds of sensitivity, and could establish a series of decreasing sensitivities toward the corpus luteum hormone i.e., myometric reaction,[3] endometrial 'lace,' implantation, traumatic deciduoma.

In studying the influence of estrogen on the manifestations of the luteal phase in the rabbit, very characteristic phenomena were observed (Courrier and Kehl, 1931a, b). Not only was the endometrial 'lace' suppressed, but an intense congestion of the uterus was produced, followed by vascular ruptures and degeneration. The horns of the uterus are hypertrophic, edematous, and hemorrhagic; their cavities contain blood and the walls show necrotic areas. The appearance is that of a violent menstruation. Before the onset of degeneration, the strong hyperemia is accompanied by a reaction of the arterial walls—the smooth muscle cells become pale and are transformed into decidual cells containing glycogen. These perivascular decidual sheaths appear normally at the point of implantation of an egg (Masquelin and Swaen, 1879); they remain well localized in the case of gestation or of traumatic deciduoma, but in our experiments the injection of estrogen in presence of progesterone made these perivascular decidual sheaths appear all over the uterus, both in the myometrium and in the endometrium. In order to produce the phenomenon just described, the estrogen has to act upon a uterus previously treated with progesterone. These processes of congestion and degeneration were found again by Leonard *et al.* (1932), by Allen (1932) and later by Gillman (1941), who used certain mixtures of estradiol benzoate and progesterone in the castrated rabbit.

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[2] It would be interesting to provoke a similar temporary suspension in the rat or mouse; there might be a retardation of implantation in these animals.

[3] From the research of Reynolds and Allen (1932) which will be referred to later.

It was desirable to measure the quantities of the two hormones that antagonize one another in the endometrium, and therefore to balance rat-units (or mouse-units) of estrogen against rabbit-units of progestin. Figures have been given in particular by Tausk *et al.* (1931), by Leonard *et al.* (1932) and by Allen (1932), etc. The great variation in biological units is well known, and hence the problem was simplified when the chemists were able to provide crystalline hormones. Having received some estrone from the first International Standard adopted in London in July 1932, we injected it into rabbits, which, following mating, began to luteinize; we again encountered all the processes described above (Courrier and Raynaud, 1933). The action was therefore due to the estrogen itself and not to impurities in the extracts. A whole series of uterine modifications were obtained according to the quantity of estrone injected. With low doses (18 μg. in 6 days following coitus) gestation could occur, but increasing quantities brought about, successively, absence of implantation accompanied by the endometrial 'lace,' temporary suspension of the 'lace,' definite suppression of 'lace,' violent hyperemia, formation of decidual perivascular sheaths, hemorrhage, and uterine degeneration.

We subsequently used estradiol and progesterone in the rabbit castrated the day after accepting the male (Courrier, 1936a). When such a female is injected with 1.5 mg. of progesterone in 5 days, a rich endometrial lace is obtained on the sixth day. If this experiment is repeated with the addition of various doses of estradiol, it is found that with 1.5 mg. of progesterone and 0.02 mg. estradiol, the lace is absent $\left(\frac{E}{P} = \frac{20}{1500} = \frac{1}{75}\right)$. Estradiol (E) has therefore neutralized 75 times the weight of progesterone (P) in the reaction producing the endometrial lace of the rabbit. Some months later Robson (1936) obtained the same results.[4]

[4] We must draw attention to results which differ from ours. Pincus (1937), in the rabbit, obtained synergism when $\frac{E}{P} = \frac{3}{420}$ and antagonism when $\frac{E}{P} = \frac{75}{420}$. Werthessen and Gargill (1945) did not find antagonism in the rabbit with $\frac{E}{P} = \frac{297}{420}$, injecting the hormones in three days.

In the conclusions of our publication of 1936a it was stated that the numerical facts resulting from these experiments should make possible an accurate measurement of the secretory activity of the corpus luteum parenchyma by the method of antagonism, i.e., by titrating it against estrogen. Allen (1937) applied the process in the following manner: in the castrated rabbit, 675 rat units of estrogen inhibited the action of 3 rabbit units of progesterone on the endometrium. He then attempted to neutralize the effect of the pregnant corpus luteum: 675 rat units of estrogen injected during the first 5 days of pregnancy did not exert any inhibiting action, but the endometrial proliferation ("lace") was absent in presence of 1000 units. Allen concluded that the corpus luteum of the rabbit produced more than 3 rabbit units (3 mg.) of progesterone during the first 5 days of its existence. However, he did not take into account, in this calculation, the physiological estrogen present in the organism. Robson (1938a) also made use of the antagonism between progesterone and estrogen in the vagina of the mouse (see below) in order to arrive at the quantity of progesterone produced during pregnancy. The relation $\frac{E}{P} = \frac{1}{75}$, in which estradiol (E) neutralizes progesterone (P) in the endometrium of the rabbit, was given above. Attention was, however, drawn to the *absolute value* of the quantities of the hormones used. The following experiment shows the importance of the absolute values (Courrier and Poumeau-Delille, 1945). Castrated rabbits were sensitized by estrogen, and, after a rest of 24 hours, treated in the following different ways during the next 5 days.

1. Some were given 0.3 mg. of progesterone daily
2. Some were given 0.3 μg. estradiol and 0.3 mg. of progesterone daily

$$\left(\frac{E}{P} = \frac{1}{1000}\right)$$

3. Some were given 3μg. estradiol and 3 mg. progesterone daily

$$\left(\frac{E}{P} = \frac{1}{1000}\right)$$

4. Some were given 30 μg. estradiol and 30 mg. progesterone daily

$$\left(\frac{E}{P} = \frac{1}{1000}\right)$$

In experiments 1, 2, and 3, the progestative lace was well developed, and there was no appreciable difference between the three groups; thus with $\frac{E}{P} = \frac{1}{1000}$, there is no antagonism in the endometrium of the rabbit.

However, with the same ratio in the fourth set of experiments, the antagonism was clear cut—the lace was poorly developed. The absolute values, therefore have great importance. When these results were published, the work done during the war by Seegar Jones and Astwood (1942) on the vagina of the rat, and that of Gillman and Stein (1942) on the uterus of the rabbit was not available to us. These workers also showed the importance of absolute values in the interactions between estrogen and progesterone. Our results agree entirely with those of Gillman and Stein. The following hypothesis is suggested in explanation of these results in the rabbit: 30 mg. of progesterone daily is an enormous dose for this female, while 30 μg. estradiol is not. It is possible that part of the 30 mg. of progesterone does not react on the uterine mucosa and hence, in the ratio of $\frac{1}{1000}$, the denominator is diminished and the ratio is not effectively reached. It is of course the *physiologically active* quantities that must be considered, since the antagonism takes place in the receptor cells.

It was stated above that estradiol can, under some conditions, neutralize 75 times its weight of progesterone in the endometrium of the rabbit, and the importance of absolute values was also pointed out. To this must be added that the interactions between estrogen and progesterone vary markedly with the receptor organs and the zoological species.

The experiment which most clearly shows the influence of the receptive tissue is that of Reynolds and Allen (1932) with the rabbit. In a study of the uterine muscle it was shown that, in all species, estrogen favors its rhythmic motility and increases its response to posterior pituitary extract. The effect of progesterone varies with the species. The writer agrees with Knaus (1930) that in the rabbit the corpus luteum hormone opposes the action of the posterior pituitary on the myometrium; there is not, as some authors believed in 1932, a specific desensin, different from the progestin acting on the endometrium. Reynolds (1931) showed clearly the antagonism between progesterone and estrogen in the myometrium of the rabbit. With Allen (1932) he did some very important experiments clearly confirming that an extract of the corpus luteum acts to oppose the uterine motility caused by estrogen. Injecting rabbits with estrogen during the first 5 days of the luteal phase (200 R.U. per day) these two authors showed that young corpora lutea neutralize the influence of estrogen on the uterine motility; but, at the same time, estrogen itself opposes the action of the corpora lutea on the endometrium, i.e., the lace is not formed. Thus, in the same animal, in the same uterus, and at the same time, the follicular hormone opposes the action of the corpus luteum hormone on the mucosa, and the corpus luteum hormone opposes the action of the follicular hormone on the musculature. This demonstrates perfectly the importance of the receptor tissue in these hormonal interactions; it is possible that the results of Reynolds and Allen may be due to the different sensitivity of the endometrium and the myometrium to progesterone in the rabbit.[5]

It should be pointed out here that Robson (1936) has studied the motility of the uterus in castrated rabbits which were given various proportions of crystalline estrone and progesterone: for $\frac{E}{P} = \frac{0.08}{0.75}$, the action of the estrone is dominant on both the muscle and mucosa; for $\frac{E}{P} = \frac{0.01}{3}$, the action of the progesterone is dominant on both the muscle and the mucosa; for $\frac{E}{P} = \frac{0.01}{0.75}$, the action of the estrone is dominant on the mucosa, and that of the progesterone on the muscle. This third experiment confirms the results of Reynolds and Allen.

Similar neutralization by progesterone of the action of estrogen on the motility of the myometrium of the rabbit was not found in the guinea pig, the mouse, or the rat, according to Siegmund and Kammerhuber (1931). Knaus found that the myometrium of women acts similarly to that of the rabbit, in that progesterone opposes the motility and renders the uterus refractory to "oxytocin." These conclusions seem important in regard to gestation and the determination of parturition; the ovarian or placental progesterone inhibits the exciting action of the estrogens on the motility of the myometrium. At the end of gestation, however, the hormonal equilibrium is modified in favor of the estrogens. However, the results of Knaus have been invalidated by Kurzrok *et al.* (1937) and by Henry and Browne (1943),[6] according to whom progesterone does not exercise a preventive action on the motility of the human uterus, and its role in the treatment of habitual abortion may not be due to a calming action on the uterine muscle, but to its influence on the endometrium. In France, Palmer and Palmer (1948) seem to agree with the ideas of Knaus. In view of these contradictory conclusions, new research is necessary on human subjects.

The influence of the zoological species must be taken into account in hormonal interactions. In the Algerian monkey, the female of which shows a characteristic menstrual cycle, and a sexual skin, the region surrounding the vulva and the anus undergoes a considerable edema under the influence of estrogen (Courrier and Gros, 1935a, b; 1937; Courrier 1936b; 1937). The experiments were done on immature castrated females, about two years old.

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[5] The importance of the receptor organ appears also in the work of Hisaw (1935–36a, 1935b) on the rhesus monkey and in that of Courrier (1937) and Courrier and Gros (1937) on the Algerian monkey; at the same time, E and P act synergistically on the uterus, and antagonistically on the sexual skin and the vagina.

[6] Henry and Browne give the complete bibliography of the problem.

(1) Nine milligrams of estradiol was injected in 15 days. The sexual skin was considerably hypertrophied. Subsequently, 5 mg. of progesterone was given daily for 12 days; the sexual skin withered rapidly and the uterus took on the characteristics of progestation.

(2) Nine milligrams of estradiol was injected in 15 days, and the sexual skin was greatly developed. Subsequently, 5 mg. of progesterone was given daily with 0.8 mg. estradiol for 12 days or longer $\left(\frac{E}{P} = \frac{0.8}{5} = \frac{1}{6}\right)$. The sexual skin withered and the endometrium again took on the characteristics of progestation.

Our experiments show that (a) the action of progesterone easily dominates that of estradiol on the sexual skin, since with $\frac{E}{P} = \frac{1}{6}$, estradiol is no longer able to act; (b) progesterone acts also on the endometrium in the presence of a strong proportion of estradiol. Certainly in the rabbit, under similar conditions, progesterone would not be able to bring the endometrium to the state of progestation. The interaction of the two hormones in the uterus is thus very different in the monkey and in the rabbit.

Hisaw and his coworkers Greep and Fevold (1935, 1936, 1937a) have pointed out that progesterone reduces the effect of estrogen on the sexual skin and the vagina of the rhesus monkey. Gillman and Stein (1939, 1941, 1942) have undertaken similar experiments with the baboon; they noted also the great differences in the estrogen-progesterone interaction between rabbit and baboon. They point out that in the baboon, 2 mg. of progesterone per day was able to neutralize, in the sexual skin, the action of 5 and 10 mg. of estradiol benzoate. It is to be assumed that 2 mg. of progesterone neutralizes the entire quantity of estrogen functional on the sexual skin, so that the surplus injected estrogen is not to be reckoned with.

Clark and Birch (1948) studied the sexual skin of the chimpanzee under the influence of combined injections of estradiol and pregneninolone. The latter possesses some inhibitory action, but it is one of the steroids which has multiple actions, although different from progesterone (see below).

It should be pointed out here that Zuckerman (1937a, b) found that estrone was capable under certain conditions of neutralizing the action of progesterone on the uterus of the rhesus monkey (500 I.U. of estrone and 10 mg. of progesterone per day or 300 I.U. and 5 mg.). Further, Corner (1938), studying the menstruation of this monkey, deduced that progesterone inhibited the action of the estrogen on the endometrium. However, Hisaw (1942a) suggests that, in this species, the two hormones work in synergism both to prevent menstruation and to produce the characteristic state of progestation in the endometrium.

The differences between the hormonal interaction in the uterus in different zoological species perhaps explain the divergent results in regard to follicular abortion, that is, the interruption of gestation due to estrogen. We have already mentioned the first work done on follicular abortion. It is certain that it is not due to the toxic action of impurities in extracts, since crystalline estrone of international standard prevents the implantation of eggs in the rabbit (Courrier and Raynaud, 1933). The problem is treated in detail in our book (1945a, pp. 274-292). In brief, the action of estrogen on gestation is very variable in different animals. In several species abortion is easier to produce at the beginning of gestation, and it is necessary to increase the dose of estrogen with the development of gestation.[7]

In the cat we found (Courrier and Gros, 1935c; Gros, 1936) that if estrogen is given in the interval between copulation and implantation, it prevents the implantation, and also neutralizes the progestational changes in the uterus. But after implantation, gestation is very difficult to interrupt with estrogen; sometimes the pregnant female enters estrus without aborting, and the newborn young show a marked genital disorder induced by estrogen which has crossed the placenta.

In the rabbit (Courrier and Kehl, 1933b; Courrier and Raynaud, 1934) the interruption of gestation is easily brought about at any moment; certainly there is no more need for estrogen on the 22nd day than on the 5th day, but rather the contrary. Parkes *et al.* (1938) came to the same conclusion in the rabbit, using ethinylestradiol and diethylstilbestrol (see the concluding section). D'Amour and his coworkers (1933, 1934), Dantchakoff (1938), and others, concluded that estrogen exerts a toxic action directly on the fetus, which becomes more resistant as it develops. Our research on the rabbit led us rather to conclude that follicular abortion is a symptom of hormonal antagonism, and that the phenomenon (considering the results of Reynolds and Allen and of Robson) takes place at the level of the endometrium and not of the myometrium.

Courrier and Raynaud (1934) showed in the rabbit that quantities of folliculin intermediate between the doses that give total abortion and those which produce no results, do produce partial abortions in which living developing fetuses and dead fetuses in the process of resorption are found side by side in the horns of the uterus. These results are similar to those of Fraenkel (1910) in which the incomplete destruction of the corpus luteum produced partial pregnancy. Further, we have shown that in the castrated pregnant rabbit all the fetuses can be kept alive with a large dose of progesterone; a smaller dose induces a partial pregnancy, in the course of which some fetuses die and others develop. This has led to the establishment of a relationship between the quantity of progesterone that acts on the uterus and the number of developing fetuses. It appears as though the estrogen injected into the pregnant rabbit acts by neutralizing a certain quantity of progesterone: partial or total hormonal antagonism gives rise to partial or total abortion.

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[7] Rat—M. G. Smith (1926), D'Amour and coworkers (1933–1934), Hain (1935), etc.; mouse—Parkes and Bellerby (1926), Burdick and Pincus (1935), Raynaud (1942), etc.; guinea pig—Kelly (1931); cat—Courrier and Gros (1935c).

It is certain that the corpus luteum itself, during pregnancy, is not a causal factor in estrogen-produced abortion; on the contrary, estrogen exerts a stimulating action on it. Further, we have succeeded in maintaining gestation in the castrated rabbit with a reasonable dose of progesterone, i.e., 3 mg. of progesterone per day allowed the normal development of six fetuses between the 12th and 22nd day. This gestation is totally interrupted by injecting in addition 0.04 mg. of estradiol in the course of each of the 13th and 14th days $\left(\frac{E}{P} = \frac{1}{75}\right)$. If 13 instead of 3 mg. of progesterone are injected with 0.04 mg. of estradiol during the 13th and 14th days $\left(\frac{E}{P} = \frac{1}{325}\right)$, total abortion still results.

Having shown that during a normal gestation in the rabbit, 30 μg. of estradiol per day, given on the 12th and 13th days, produces abortion without fail, the following experiment was performed; a pregnant rabbit was given 30 mg. of progesterone per day from the 10th to the 18th day, then injected with 30 μg. of estradiol daily during the 12th and 13th day $\left(\frac{E}{P} = \frac{1}{1000}\right)$. The result was total abortion. In view of the results already mentioned concerning absolute values, $\frac{E}{P} = \frac{1}{1000}$ leads to antagonism when $E = 30\ \mu g.$ and $P = 30$ mg. Up to now it has been impossible to demonstrate that an abortive dose of estrogen loses its toxicity in presence of sufficient progesterone. It is hoped that some of our students will take up the question in the mouse, for example.

Another argument in favor of the idea that follicular abortion is a manifestation of hormonal antagonism is furnished by a study of the eggs of the rabbit before implantation. Premature destruction of the corpus luteum brings about involution of the blastocysts toward the 4th day, that is, before implantation (Ancel and Vintemberger, 1929; Corner, 1928). It appears that the life of the free blastocyst requires some special uterine secretion which depends upon the hormone of the corpus luteum. Further, Pincus and his coworkers, Burdick (1935) and Kirsch (1936), have shown that estrogen[8] in low dosage causes the same effect as suppression of the corpus luteum: the blastocysts die toward the 4th day.

Since these workers have shown that estrogen has no direct toxic action on the free egg, it appears as though the injection of estrogen interferes with the action of the corpus luteum on the uterus. Perhaps our results of 1928, in which certain doses of estrogen oppose implantation without opposing the endometrial lace, can be better understood in this way.

If the interrelations between estrogen and progesterone are responsible for the mechanism of follicular abortion, then the primates doubtless do not behave like the rabbit described above. Reference may be made to the unique observation of Marker and Hartman (1940) in *Macacus rhesus:* they injected 50 mg. estrone per day between the 78th and 98th day of gestation and later found a macerated fetus whose size was that which would normally have been reached on the 100th day. In women, several workers have shown that estrogen in massive doses cannot interrupt a pregnancy undergoing normal development. It may be supposed that the living placenta secretes a locally-acting progesterone which opposes the abortive action of estrogen. When the egg is dead, the estrogen associated with posterior pituitary extract facilitates its expulsion by muscular action.

In discussing in the preceding pages the interactions in the uterus between estrogen and progesterone, special mention has been made of the rabbit, the guinea pig, the monkey and the cat. Research has also been undertaken with the rat and the mouse.

Votquenne, in our laboratory, using the rat (1934, 1936), has shown that estrogen clearly opposes the deciduoma of Loeb produced by the hormone of the corpus luteum, as it does in the guinea pig (Courrier, 1930a, b) and in the rabbit (Brouha, 1934). Astwood (1939a) has also obtained an inhibiting effect of estradiol on the deciduoma of the castrated pseudopregnant rat, caused by progesterone. Rothchild *et al.* (1940) have also studied the rat. The females were made pseudopregnant by stimulating the cervix of the uterus; on the 4th day, they were castrated and the uterus traumatized by a needle. They were then injected from the 4th to the 8th day with progesterone, alone or together with estradiol, and the uterus examined on the 9th day. Giving a constant quantity of progesterone, and varying the doses of estradiol, a synergism was observed for the weak doses (deciduoma more voluminous), and an antagonism for the strong doses (deciduoma small or absent). The synergism was obtained with 3 mg. of progesterone and 0.15 μg. estradiol $\left(\frac{E}{P} = \frac{1}{20{,}000}\right)$, antagonism with 3 mg. of progesterone and 3 μg. of estradiol $\left(\frac{E}{P} = \frac{1}{1000}\right)$.

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[8] Pincus (1937) makes a distinction between estrone and estradiol: the former inhibits the growth of the free egg and the development of the uterine lace, while the latter inhibits the lace without inhibiting the growth of the free egg.

We recently asked our collaborator, F. Morel (1948), to study the mouse, because Hooker and Forbes stated in 1947 that the estrogen-progesterone interaction is very different in the mouse uterus from that in the rabbit, showing neither synergism nor antagonism. Morel castrated adult mice and treated them as follows:

> (a) Estradiol 0.02 μg. per day for 2 days; then 0.5 mg. progesterone per day for 6 days. The uterus was traumatized by a wire on the 3rd day of the treatment with progesterone, and, at the end of the experiment, 25 deciduoma were found in 26 experiments.
>
> (b) The experiment was repeated, but beginning with the day of the traumatization 5 μg. of estradiol per day was added to the progesterone $\left(\frac{E}{P} = \frac{1}{100}\right)$. In 26 experiments, 23 animals showed no trace of deciduoma. Antagonism is therefore shown in the mouse: estrogen is able to prevent the action of progesterone on the mucosa of the uterus as in the rabbit, guinea pig, cat, and rat.

Before ending this section the influence of the interactions on the histochemistry of the uterus must be mentioned. Variations of the alkaline phosphatase of the endometrium have been shown by Atkinson and Engle (1947). Variations in glycogen as a function of varying quantities of the two hormones have been demonstrated by Hisaw and Greep (1938) in the monkey, and by Rothchild and coworkers (1940) in the rat. This is an interesting point of view to follow, and further studies on women should be encouraged.

IV. ANTAGONISM IN THE VAGINA

It has already been mentioned that Smith, working with rats (1926), and Parkes and Bellerby (1926, 1927a, b) with mice, have shown that follicular extract is less effective on the vaginal epithelium when the ovary contains corpora lutea. This phenomenon has subsequently been studied by many workers. The bibliography can be found in the chapter by Hisaw (see E. Allen, 1932). The following are among the first papers published in France: Gley (1928), Cotte and Pallot (1928), Brouha and Simonnet (1929a), Goormaghtigh and Armerlinck (1929), Lipschütz and Adamberg (1929).

There was at first some confusion among certain workers, but in 1928 we showed that two different actions of the corpus luteum hormone must be distinguished: 1) it can exercise a sterilizing action, preventing ovulation and estrus; 2) it can directly oppose the keratinizing action of estrogen on the vagina. This latter property is of interest here, since it involves interaction between the two hormones on the vaginal receptor. This particular interaction has been widely discussed, but in some instances the corpus luteum has been falsely implicated (Loeb and Kountz, 1928). Courrier *et al.* (1929a, b) have shown essentially that in the pregnant guinea pig whose pregnancy is normal after castration, injection of follicular hormone has no effect on the vagina in the absence of the ovary; the ovarian corpus luteum could not, therefore, be paralyzing the reaction in this case, and presumably the placenta must intervene.

The action of the corpus luteum hormone in neutralizing the effect of estrogen on the vagina was accepted by some and rejected by others, including among the latter Brouha and Simonnet (1929b), Angelwitz and Sterling (1930), and Desclin and Dessiennes (1940).

We now know that progesterone is able to antagonize the keratinizing action of estrogen on the vaginal epithelium, but only under favorable experimental conditions. In order to study this antagonism, we have analyzed, at the same time, in the same organism, the functional value of the two hormones—estrogen, by its vaginal action, and progesterone, by its action on the deciduoma of the uterus. This method was used with the guinea pig (1930a, b), and in 1934, my student Votquenne performed demonstration experiments on the rat in the same way. A certain amount of estrogen, which provokes keratinization of the vagina in the castrated female, becomes inactive in the presence of the corpus luteum of lactation, the activity of the latter being indicated by the formation of the traumatic deciduoma. If the amount of injected estrogen is increased, then in some doses the deciduoma of Loeb no longer appears, and the vagina is keratinized. Thus it follows either that progesterone has an anti-estral action or that estrogen has an anti-progesterone action.

In 1934, de Fremery, Kober, and Tausk demonstrated that progestin opposed the action of estrin on the vagina in the castrated mouse, and in 1935, Allen and Meyer obtained very interesting results with the same animal: 3 R.U. of follicular extract given in 8 days provoked vaginal keratinization; 4 rabbit units of "progestin" (free from estrogen) gave no reaction in 8 days. However, 3 R.U. of estrogen with 4 rabbit units of progestin injected at the same time, caused intense vaginal mucification. This vaginal mucification is not due to any specific hormone of the corpus luteum, but results from the conflict between the two hormones on the epithelial cells of the vagina; the appearance of mucification indicates that progesterone opposes the keratinizing action of the estrogen. Further, Patel indicated in 1930 that progesterone reduces the activity of estrogen on the vagina, and that estrogen in weak doses stimulates mucification.

The work of Klein (1937, 1938) on the hamster should also be mentioned. It shows that the addition of progesterone to doses of estrogen large enough to provoke keratinization causes an intense mucification of the vagina. Hisaw *et al.* (1937a) also demonstrated that in the monkey progestin opposed the keratinizing action of estrin on the vagina, without resulting in any other special cellular product—there is no mucification.[9]

These experiments led up to a quantitative study of the conflict of the two hormones on the vaginal epithelium, making use of crystalline substances.

Selye *et al.* (1936) administered 30 μg. of estrone and 400 μg. of progesterone per day to castrated rats. The experiments lasted 20 days, during which time the vaginal smear was almost always negative and the vaginal epithelium was mucified $\left(\frac{E}{P} = \frac{30}{400} = \frac{1}{13}\right)$.

Courrier and Cohen-Solal (1937a) administered the two hormones in a single injection to castrated rats; 20 μg. of estradiol gave 30 positive vaginal smears in 33 females. Upon adding 4 mg. of progesterone, there were still 5 positive smears from 9 animals, while with 8 mg. there was no positive response in any animals.

Robson (1937) showed that in the mouse 750 μg. of progesterone are necessary to prevent keratinization caused by 0.2 μg. of estrone.

Freud (1937) was able to produce vaginal mucification in the castrated mouse by daily injections of 10 to 50 μg. of estrone with 1 to 2 mg. of progesterone.

In 1937, Korenchevsky and Hall, using the castrated rat, obtained the following results: 30 μg. of estrone per day caused vaginal keratinization; 30 μg. of estrone with 400 μg. of progesterone gave slight mucification; 1 μg. of estrone with 1500 μg. of progesterone caused mucification characteristic of pregnancy, after several days' treatment.

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[9] Also in the "uterus masculinus" of the prostate of the monkey, progesterone can oppose the epithelial action of estrogen without resulting in any special cellular product (Zuckerman and Parkes, 1936).

But in 1940, Desclin and Dessiennes were unable to neutralize the keratinizing action of estradiol on the vagina of the castrated mouse by means of progesterone; they injected simultaneously 1 μg. of estradiol benzoate, and 1 mg. of progesterone.[10] Courrier and Poumeau-Delille (1944) reported that to show the antikeratinizing action of progesterone clearly it is preferable to administer the two hormones, not as a single injection, but over several consecutive days, and to wait until equilibrium is established.

The following are some typical experiments in the castrated rat:

(1) 2.5 μg. estradiol per day.
Vaginal smear: 1st, 2nd and 3rd day: negative (leucocytes)
4th, 5th, 6th, 7th, and 8th day: positive (single cornified cells)

(2) 2.5 μg. estradiol per day + 250 μg. progesterone per day $\left(\frac{E}{P} = \frac{1}{100}\right)$. Keratinization appears on the 4th day and persists. There is no antagonism.

(3) 2.5 μg. estradiol + 1.25 mg. progesterone per day $\left(\frac{E}{P} = \frac{1}{500}\right)$. The result varies with the animal.

In some, the vaginal smear is negative from the 1st to the 8th day; in others, the smear is positive on the 4th and 5th days and becomes negative again at the beginning of the 6th day. There is, therefore, in some animals a temporary vaginal keratinization, antagonism reappearing thereafter. This temporary keratinization can also appear with 2.5 μg. of estradiol and 5 mg. of progesterone $\left(\frac{E}{P} = \frac{1}{2000}\right)$, and leads to error if one does not continue the experiment long enough to reach equilibrium.

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[10] In our laboratories, Morel recently subjected castrated mice to different treatments for 4 days: the results varied with the relationship E/P.

Estradiol per day, μg.	*Progesterone per day, mg.*	*Vagina*
0.02	0	Keratinization
0.02	0.5	Mucification
0.5	0	Keratinization
0.5	0.5	Polystratification and extravasation of leucocytes
5	0	Keratinization
5	0.5	Keratinization

It should be mentioned here that in February, 1942, injecting castrated rats with only 5 mg. per day of desoxycorticosterone acetate (a compound that also produces the characteristics of pregnancy), we obtained vaginal keratinization on the 3rd day. However, it lasted only a short time, and after its disappearance, the substance opposed the keratinizing action of estradiol. Del Castillo and Di Paola had found this antagonism also (1940).

Progesterone can also produce keratinization, as shown by Gros (1942) and Clarke and Selye (1943). The writer (1945b) noted that this keratinization is of short duration and gives way to the antikeratinizing action when in the presence of estrogen. It is thus necessary, when studying the progesterone-estrogen interaction on the vagina, to carry on the experiments for several days. This transitory keratinization will be referred to again in the discussion of pregneninolone, a steroid that is clearly more polyvalent than progesterone.

Only after the war were the important results of Seegar Jones and Astood (1942) available to us. These workers injected castrated rats with varying quantities of estradiol and progesterone for about 10 days. The result on the vagina depended on the absolute amounts of the injected hormones. They noted in particular that when the dose of progesterone was 1 mg. per day, estradiol no longer guaranteed a continuous vaginal keratinization, whatever the quantity administered $\left(\text{for example } 100\ \mu\text{g. per day: } \frac{E}{P} = \frac{1}{10}\right)$. The writer confirmed these results, and Alloiteau (1950) has recently found that with 1250 μg. of estradiol and 1250 μg. of progesterone $\left(\frac{E}{P} = \frac{1}{1}\right)$, the rats showed vaginal mucification.

As the amount of estrogen is increased, the amount of progesterone necessary for antagonistic action does not increase proportionately. It may be supposed that at some point the amount of estrogen which can be functionally active is exceeded; part of the injected estrogen then no longer enters into the physiological picture. If 1 mg. of progesterone neutralizes the entire functional dose of estrogen that acts on the vagina, it is evident that this 1 mg. will oppose any amount of estrogen that may be given. This hypothesis makes it possible to explain the experiments made earlier on the uterus of the rabbit, which demonstrated the importance of the absolute amounts.

Hormonal antagonism between progesterone and estrogen also exists on the vaginal receptor tissue of the rabbit, as was shown by Jost (1944) in our laboratory. This experiment is an interesting one to mention because the vaginal epithelium of the rabbit behaves in a special way, i.e. (as we showed in 1926) it is always covered by a single layer of cells that undergo an intense mucification, at the time of estrus, under the influence of estrogen. Jost established the fact that certain doses of progesterone radically oppose the mucus-producing action of the estrogen. There is thus in the rabbit no special cooperation of the two hormones to lead to mucification, and antagonism is indisputable.

Hormonal interactions also affect the vagina in women. With women in menopause, Shorr (1940) noted that progesterone opposes the keratinizing effect of estrogen on the vaginal epithelium. During human pregnancy, Hall (1945) recommended a treatment with progesterone to prevent complications due to an upset hormonal equilibrium, a disturbance which is evidenced by a very pronounced vaginal keratinization.

Finally, it should be mentioned that Kamell and Atkinson (1948) have begun a study of the effect of the two female hormones individually or together on the histochemistry of the vaginal epithelium in the mouse, with special reference to ribonucleic acid and alkaline phosphatase.

The mechanism by which progesterone opposes the keratinizating action of estrogen is still unknown. We will only note here that Hechter *et al.* (1940) found that atropine also stopped the action of estrogen on the vagina by inhibiting the induced hyperemia.

V. ANTAGONISM IN OTHER RECEPTORS

Antagonism between the two female hormones appears not only in the uterus, in the sexual skin and in the vagina, but also in other receptor organs.

In women, the glands of the cervix secrete the cervical mucus which has long been studied by gynecologists (since Tyler Smith in 1855). The important work of Seguy and Vimeux (1933) brought out the cyclical modifications of the cervical mucus. Between the 10th and 15th day after the beginning of menstruation it becomes abundant and clear, its viscosity decreases considerably ("glaire filante") and it is now permeable to spermatozoa. The secretion of such mucus can be produced in ovariectomized women by means of estrogen (Moricard, 1936). Palmer (1941, 1944) found that the appearance of this "glaire filante" at the cervical orifice was dependent upon estrogen. It disappears abruptly in the course of the normal cycle, due to inhibition by progesterone of the secretory action of estrogen on the cervical glands. Palmer showed that 2 mg. of estradiol benzoate given to castrated women resulted in a "glaire filante" characteristic of the follicular phase of the menstrual cycle, but he also makes the following important statement: 10 mg. of progesterone injected at the same time as 2 mg. of estrogen opposes the action of the latter, and the "glaire filante" is no longer apparent. Progesterone has thus neutralized the action of estrogen on the secretion of the mucous glands of the cervix in women, at the ratio $\frac{E}{P} = \frac{2}{10}$. Palmer considered that abnormally prolonged persistance of the "glaire filante" at the cervical opening is probably due to an insufficiency of corpora lutea.

These interesting results have been confirmed by Abarbanel (1946) who found that from 5 to 20 mg. of progesterone overcame the effects of 0.5 to 1 mg. of estradiol.

The work of Palmer and Abarbanel might perhaps be used to evaluate the amount of estrogen physiologically active in the normal woman during the estrus cycle, ascertaining the amount of progesterone which is necessary to prevent the appearance of the cervical "glaire filante."

It is known that estrogen causes both morphological and functional changes in the anterior pituitary. Progesterone is capable of modifying these influences. Brooksby (1938), using castrated female rats, found that if progesterone was added to the estrogen, it reinforced the action of the latter on the pituitary, which accordingly underwent an increased hypertrophy. Later workers have not found such synergism, but rather the contrary. Thus, Selye (1940b), Schilling and Laqueur (1941), and Smith (1944), have shown that the hypertrophic action of estrogen on the pituitary and the adrenals is directly opposed by progesterone. Bradbury (1946) found that progesterone opposes the diminution in the amount of pituitary gonadotropin caused by estrogen.

Smith and Smith have formulated a theory to explain the effect of progesterone—it is not the estrogen itself that stimulates the pituitary but the "inactive" products derived from the metabolism of the estrogen (lactone); progesterone opposes this "inactivation" and promotes the excretion of the estrogen (see below).

The work of Lipschütz and his pupils (1946, 1948) on the intrasplenic grafting of the ovaries of a castrated guinea pig proves, according to the authors, antagonism between the two female hormones in their influence on the ovary. The grafted ovaries in the spleen develop cystic and hemorrhagic follicles. Estradiol prevents this reaction and instigates a marked luteinization. Progesterone, when added to the estradiol, opposes the luteinizing action of the latter, but hemorrhagic follicles do not appear.

The idea of antagonism between estrogen and progesterone is also found in the work of Turner on the mammary glands. In studying the cause of the initiation of lactation Meites and Turner (1942, 1948) found that estrogen instigates the secretion of pituitary prolactin, but progesterone opposes this action up to the moment of parturition. At this time the estrogen becomes dominant. Prolactin is secreted in abundance, and milk production is achieved. These two workers have studied the effects of different proportions of estrone and progesterone on the content of prolactin in the pituitary. In the guinea pig, when the proportion of progesterone to estrone is 2000:1, progesterone totally neutralizes the stimulating effect of estrone on the hypophyseal prolactin. When the proportion is 250:1, progesterone no longer exercises any such opposition. Nevertheless, the role of absolute amounts should be studied in this connection.[11] The ideas of Meites and Turner on the causes of the initiation of lactation are not endorsed by Walker and Matthews (1949), who have not been able to prevent milk production in rats by injecting them with progesterone before parturition. More research is needed on this problem.

In connection with lactation, the influence of certain proportions of estrogen and progesterone on the continuation of milk production must be mentioned. Bibliographical references may be found in a recent article by Masson (1948); in the castrated and nursing female rat, a ratio of $\frac{E}{P} = \frac{10}{10{,}000}$ stops the secretion of milk, while each of the two hormones given separately produces no effect. The hormonal combination acts synergistically to cause proliferation of the acini of the mammary glands, and the secretory activity can no longer continue.

Antagonism between estrogen and progesterone has been indicated also in reference to the prostate by Zuckerman and Parkes (1936). Estrogen has a characteristic action on the prostate of the monkey, producing in particular the epidermization of the epithelium of the "uterus masculinus" (Courrier and Gros, 1935a, b; Parkes and Zuckerman, 1935). This epidermization, due to estrone, can be reduced by the presence of progesterone (50 μg. of estrone and 1 mg. of progesterone).

By giving estrogen over a long period, it is possible to bring about certain pathological transformations, which have been treated, sometimes with success, by means of progesterone. Thus in the rhesus monkey, Hisaw (1935b), by the use of progestin, prevented pathological modifications of the cervix due to prolonged administration of estrin.

Dessau (1937), using the guinea pig, showed that progesterone prevents glandular-cystic hyperplasia of the uterus and epidermoid metaplasia of its epithelium caused by estrone or estradiol. Korenchevsky and Hall (1938) induced metaplasia of the epithelium of the rat uterus, which became stratified, by administering 30 μg. of estrone or estradiol per day for 4 weeks. The addition of 400 μg. of progesterone per day totally prevented this metaplasia.

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[11] In their publication in 1948, Meites and Turner indicate that progesterone may also be able to oppose the inhibiting effect of estrone on the growth of the organism.

Lacassagne in 1937 hoped to use this hormonal antagonism in his research on experimental carcinogenesis of the mammary glands of the mouse. He found it impossible to neutralize the tumor-producing action of estrogen on the breast of the R_3 stock by means of progesterone. However, the quantity of progesterone he used was low $\left(\frac{E}{P} = \frac{50}{100} \text{ or } \frac{50}{200}\right)$.[12]

Heimann (1945) claims to have reduced the frequency of spontaneous mammary tumors in R_3 mice with progesterone, but this has not been observed by Loeser (1941) nor by Burrows and Hoch-Ligeti (1946).

Prolonged treatment by estrogen leads to the development of fibroids (conjunctivo-muscular proliferation) in the uterus and the peritoneum of the guinea pig (Nelson, 1937; Allen, 1937; Moricard and Cauchoix, 1938; Lipschütz *et al.*, 1938). Lipschütz and his pupils (1939, 1941, 1947) have shown that progesterone prevents the appearance of these uterine and extrauterine proliferations. Such results justify the tentative treatment by progesterone of uterine fibromyomas in women, but the first published results are not encouraging (see for example Goodman, 1946; Segaloff *et al.*, 1946).

VI. SYNERGISM BETWEEN THE TWO FEMALE HORMONES

It was stated at the outset that estrogen and progesterone are able to work together under certain conditions, and it was indicated that the idea of such a synergism is supported by Hisaw and his collaborators, Weichert (1928) and Leonard (1930). In order for a corpus luteum extract to be able to bring the uterus of a castrated female into a progestational state, previous estrogen sensitization is necessary. This fact was verified by many workers, and it is known that under normal conditions, progesterone acts after estrogen.

However, experiments have subsequently shown that the previous injection of estrogen is not absolutely indispensable. Hisaw *et al.* (1937b) showed that progesterone was able to bring the endometrium of a rabbit or of a monkey into a progestational state, although the animal had been castrated for some time previously and was not sensitized by estrogen. However, in this case much more progesterone was necessary. This result was confirmed by Selye (1940a) and by

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[12] With testosterone, Lacassagne and Reynaud (1939) obtained antagonism to the appearance of spontaneous mammary cancers in the R_3 mice. But testosterone neutralizes the keratinizing action of estrogen on the vagina more effectively than progesterone.

Rothchild *et al.* (1940), who obtained a deciduoma in rats which had been castrated for a considerable length of time, by giving a strong dose of progesterone alone. Gillman and Stein (1942) found, in the rabbit not previously sensitized by estrogen, a certain progestational reaction of the endometrium caused by progesterone, but this reaction did not appear to be augmented by increasing the doses of progesterone.

If the preliminary injection of estrogen is not absolutely indispensable, it is nevertheless certain that estrogen prepares the uterus for the subsequent action of progesterone, and that it permits great economy of this hormone. This is certainly a case of synergism. Is this synergism, in the case of successive action, produced when the two hormones act simultaneously?

Hisaw has believed in the existence of such a synergism since 1930, but his experiments did not demonstrate its existence until 1935–36. He injected a rhesus monkey with estrogen and corpus luteum extract at the same time, and showed that the uterus was larger with the hormones together than with either one of them separately. Such a uterus is in the progestational state. Shortly following these experiments, several workers were able to show that estrogen, acting at the same time as progesterone, was capable of reinforcing the action of the latter.

Allen and Heckel (1936), W. M. Allen (1937), Pincus (1937), Ostergaard (1937), and Courrier and Kehl (1937), using the rabbit, upheld the idea of the synergism of the two hormones in acting on the uterus. For example, immature castrated rabbits were sensitized with estradiol (15 μg. in 6 days), and then divided into two groups: the first received only 250 μg. of progesterone per day, the second received 250 μg. of progesterone plus 0.3 μg. of estradiol per day; the treatment lasted 15 days. In the second group, the uterus was larger, and the lace characteristic of progestation was still present whereas it had already completely disappeared in the first group.

In other experiments, castrated rabbits not sensitized by estrogen were injected at the same time with 0.8 μg. of estradiol and 250 μg. of progesterone per day. The uterine lace was developed, while it did not appear when 250 μg. of progesterone alone was used.

Korenchevsky and Hall (1937), with $\frac{E}{P} = \frac{1}{1500}$, obtained marked synergism for the rat uterus in which the state of progestation was clearly evident.

Hisaw *et al.* (1937a) and Engle and Smith (1938), working with the rhesus monkey, also demonstrated cooperation between the two hormones on the uterus, of which the overgrowth was more marked, and the progestational state more typical, when estrogen was added to progesterone. As a matter of fact, it is easier to demonstrate synergism in the monkey than in the rabbit, because in the latter estrogen easily opposes the action of progesterone on the endometrium; the dose of estrogen which will lead to synergism must be very small. In the monkey this is not true; progesterone acts on the endometrium in the presence of large amounts of estrogen. It easily dominates the action of estrogen on the sexual skin.

Synergism can easily be shown during gestation (Courrier and Kehl, 1938a, b; Courrier and Jost, 1939a). In the rabbit, castration always interrupts gestation. The castrated pregnant rabbit, given 5 mg. of progesterone per day, showed normal pregnancy. If it was given only 0.5 mg. of progesterone, all the embryos were resorbed. With intermediate doses, partial gestation was obtained. If the castrated pregnant rabbit was simultaneously injected with estrogen and progesterone, antagonism (see above) or synergism could be obtained at will. It was possible to show synergism, in particular, by giving 0.5 mg. of progesterone per day, with 0.66 μg. of estradiol $\left(\frac{E}{P} = \frac{1}{750}\right)$, or better, with 0.33 μg. of estradiol $\left(\frac{E}{P} = \frac{1}{1500}\right)$. In this case living, developing fetuses were obtained, while with 0.5 mg. of progesterone alone, gestation was always interrupted. All the information can be found in the paper of our coworker Jost (1939).

Under normal conditions, a close endocrine collaboration appears to exist between estrogen and progesterone in pregnant females. The arguments in favor of this collaboration have been enumerated in our book on the endocrinology of pregnancy (1945a).

In the pregnant castrated golden hamster, Klein (1938) obtained a similar synergism. With 0.2 mg. of progesterone and 1 μg. of estrone pregnancy proceeds. With 0.5 mg. of progesterone alone, it is not maintained. It is probable that the ratios of E:P that produce synergism are not the same in the rabbit, the hamster and the rat (Rothchild *et al.*, 1940). It has already been indicated that these latter workers have brought about a clear case of synergism in their experiments on the deciduoma of the rat. Weak doses of estradiol added to progesterone increased the size of the deciduoma, while strong doses caused it to decrease in size (i.e., synergism or antagonism, as in the castrated pregnant rabbit).

A typical example of cooperation between the two female hormones is shown in experiments on the menstruation of the rhesus monkey. From the many papers which have appeared on this subject may be selected that of Hisaw and Greep (1938). It is known that the menstruation which follows a treatment of the castrated monkey with estrogen can be retarded by estrogen itself, but more easily by progesterone. The most successful method, however, consists in administering the two hormones at the same time $\left(\frac{E}{P} = \frac{2}{250}\right)$, and this ratio leads to a clearly defined state of progestation in the endometrium. Thus, in order to retard this post-estrogenic menstruation, 2 μg. of estradiol or 0.25 mg. of progesterone per day produce no action, but 2 μg. of estradiol with 0.25 mg. of progesterone given simultaneously suffice to induce a very marked retardation.

Such results must certainly lead practitioners to seek for an effectual ratio of E:P in their hormonal treatments for women. Some tentative attempts have been made by Portes and Varangot (1941), by Zondek (1942) and by Vaux and Rakoff (1945) using a combination of estrogen and progesterone, to treat recurrent abortion. It appears that great efforts are still necessary in order to determine both the absolute amounts and the ratio of E/P for the rational treatment of women.

Some recent research on the implantation of eggs in the uterus also supports the idea of an endocrinal cooperation between estrogen and progesterone. Kehl and his student Chambon (1949) considered that experimental deciduoma and ovo-implantation in the rabbit necessitated combined action of the two hormones in well-determined amounts. It was a question of synergism, not of a sparing action but of necessity, since progesterone alone in the rabbit would be insufficient to induce the experimental deciduoma, even in very large doses. The young trophoblast of the ova seems to be able to provide the necessary estrogen at the time of implantation.

The action of estrogen must be borne in mind in the case of ovo-implantation retarded by nursing (as in the rat and mouse). Discussion of this phenomenon can be found in the author's book (1945a). It is now known that ovo-implantation can be effected without retardation, in the nursing female rat, if weak doses of estrogen are injected (Krehbiel, 1941; Weichert, 1942; Bloch, 1948). Morel showed recently in our laboratory, that a single injection of 0.01 μg. of estradiol, given on the 5th day, prevents the retardation of ovo-implantation in the nursing mouse. Lactation is followed by endocrinal disturbances that are particularly noticeable in the vagina, e.g., the absence of vaginal mucus in the pregnant nursing female (Selye *et al.*, 1935; Lyon, 1939; Klein and Mayer, 1944). It seems that there may be a deficiency of estrogen, because it is diverted toward the breast and acts on the nursing young (Courrier, 1930d).

Estrogen may work together with progesterone to produce the normal implantation of eggs. Chambon (1949a, b) in recent research, experimentally obtained retardation of the ovo-implantation in castrated rats 24 hours after copulation, when they were given insufficient amounts of progesterone. A daily dose equal to or greater than 0.50 mg. of progesterone assured ovo-implantation at the normal time, but with 0.25 mg. per day, implantation was retarded. This retardation may be overcome if, with the 0.25 mg. of progesterone, 0.16 μg. of estradiol is given at the same time. If this result is confirmed it will certainly be a case of hormonal synergism $\left(\frac{E}{P} = \frac{1}{1500}\right)$.[13] Thus in this case once more "physiological processes take place within narrow quantitative hormonal limits," because if estrogen is too abundant in the rat, ovo-implantation does not take place (see above).

The study of hormonal interaction on the mammary receptor has led to important results. In some species (guinea pig, cow, and goat), estrogen alone is capable of causing the complete development of the milk ducts and acini. In others (rabbit, mouse), estrogen alone can only produce some of the ducts; complete development requires the synergistic action of estrogen and progesterone.

However, it must be noted that even when the amount of estrogen is sufficient, it is preferable to add progesterone to induce normally harmonious development, since estrogen alone may produce cystic formations which disappear in the presence of progesterone. In other words, to obtain an integrated and harmonious development of the ductsand acini analogous to that found during gestation, it is necessary that estrogen and progesterone act in well determined proportions and in the presence of the pituitary. While it is impossible to cover all of the literature here, a few papers may be mentioned. In 1941, Lyons and McGinty, Scharf and Lyons, working on the immature male rabbit, subjected it to the following treatments:

> 12 γ of estrone injected alone produced after some weeks dilated ducts without acini;—12 γ of estrogen together with 1 mg. of progesterone gave clear-cut development of both ducts and acini;—12 γ of estrone with 8 mg. of progesterone gave a less marked development than in the preceding case;—24 or 96 γ of estrone with 1 mg. of progesterone gave the best development, similar to that in pregnancy.

Thus the most favorable synergistic proportions do not correspond to our figures on the pregnant female rabbit.

In 1942, Mixner and Turner, studying the formation of acini in the castrated female mouse, obtained the following results after 10 days' treatment:

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[13] According to Lyon and Allen (1943), in a nursing nonpregnant rat a traumatic deciduoma can be obtained 5 days after parturition. To obtain a second deciduoma on the 10th day it is necessary to inject a little estrogen.

> with estrone alone, there were no acini; with progesterone alone (total dose 6 mg.) 60% of the animals had acini; with a total of 1 mg. of progesterone and 13 γ of estrone, 60% of the animals had acini; with a total of 1 mg. of progesterone and 120 γ of estrone, 33% of the animals had acini; with a total of 1 mg. of progesterone and 240 γ of estrone there were no acini.

A well marked synergism is therefore obtained with 1 mg. of progesterone and 13 μg. of estrone, but it disappears with increasing doses of estrone.

In Lacassagne's laboratory, Chamorro (1947) continued the experiments of Lyons and his coworkers on the immature male rabbit, and concluded that progesterone even in a strong dose always produces synergism when added to estrogen. It is well-known that Lacassagne could not neutralize the carcinogenic action of estrogen on the breast by means of progesterone.

It follows from the above that the total and harmonious growth of mammary tissue is obtained only with definitive proportions of the two hormones, and that progesterone prevents the formation of cysts due to estrogen. It should be remembered that some ratios of E:P inhibit the secretion of milk and cause the proliferation of acini.

It has been shown that the joint action of the two female hormones can determine mating. The females of different species do not behave in a uniform manner. In all species, estrogen produces heat to a greater or lesser extent, but in some, namely the goat and sheep, progesterone opposes this action (Fraps *et al.*, 1946). In others, on the contrary, progesterone reinforces the action of estrogen; this synergistic action is found in the guinea pig (Dempsey *et al.*, 1936), in the rat (Boling and Blandau, 1939; Astwood and Dempsey, 1941; Beach, 1942), in the mouse (Ring, 1944), and in the golden hamster (Frank and Fraps, 1945).

Dempsey *et al.* (1936) have clearly shown that progesterone, injected into the castrated guinea pig 36 to 48 hours after estrogen, increases the per cent of positive responses to the copulation reflex and diminishes the time of latency, i.e., the interval between the last hormonal injection and the manifestation of heat. In the hypophysectomized rat, Astwood and Dempsey (1941), with estrogen alone, obtained copulation in 48 hours in 50% of the treated animals; with progesterone given 36 hours after estrogen, they obtained copulation in 4 hours in 95% of the animals.

These results suggest to some workers that in certain species the normal estrus is the result of the cooperation of the two hormones, and it is accepted that progesterone is already secreted by the ripe follicle before it has ruptured (Astwood, 1939b).

The interplay of the two female hormones also takes place in the experiments dealing with the opening of the pubic symphysis in the guinea pig. A detailed discussion of the remarkable modifications of the pelvic girdle which, in certain mammals, facilitate birth, will be found in the accompanying article by Hisaw and Zarrow. Hisaw has made clear the hormonal cause of these modifications: a specific hormone, relaxin, opens the symphysis of the guinea pig after estrogen sensitization. It is possible to obtain opening of the symphysis also by a combined treatment with estradiol and progesterone, but Hisaw (1942b) has shown that in this case the hormones act through the intermediary of the uterus. Courrier and Marois (1947) have confirmed this statement, a small amount of healthy uterine mucosa sufficing to make the estrogen and progesterone capable of opening the symphysis of the guinea pig. Marois (1948) noted that hormonal synergism still takes place if the estradiol is given subcutaneously and the progesterone locally in the uterus; much less progesterone is needed in this case.

In the same castrated guinea pig, the formation of the traumatic deciduoma and the opening of the pubic symphysis in response to a mixture of estrogen and progesterone have been compared in one and the same animal (Courrier and Poumeau-Delille, 1942). This seems important, since in normal gestation, the decidual reaction takes place at the beginning, and the opening of the symphysis at the end of the process. Marois (1949) has verified the result that some combinations of E/P, antagonistic for the deciduoma, are synergistic for the opening of the symphysis.

In this chapter clear, precise examples have been given of hormonal synergism between estrogen and progesterone. Hypotheses have been formulated to explain the mechanism of this synergism. After having described the synergistic action of estrogen and progesterone on the growth of the free blastocyst in the uterus and the development of the endometrial lace in the rabbit, Pincus (1937) proposed the following explanation: estrogen and progesterone are destroyed in the organism by the same enzymatic system. If this system is saturated with estrogen, progesterone will be destroyed more slowly, and hence its action will be prolonged. It has also been suggested that estradiol can assist the action of progesterone because it increases the circulation of blood in the uterus (Astwood, 1938; Reynolds, 1939). But such hypotheses do not explain why synergism is sometimes transformed into antagonism with the increase of estrogen.

VII. PARTICULAR INTERACTIONS BETWEEN ESTROGENS AND PROGESTERONE

In this section the two special aspects of the problem with which we are most concerned will be treated briefly:

(1) The influence of progesterone on the metabolism of estrogens,

(2) The action of estrogens on the ovarian corpora lutea and on the excretion of pregnandiol.

The work of Smith and Smith and of Pincus on rabbits and women has clearly shown that progesterone intervenes in the metabolism of estrogens. (For a bibliography see the review by the Smiths (1948).) Progesterone facilitates the transformation estradiol → estrone → estriol; it opposes the transformation of estrogens into oxide derivatives[14] that are no longer estrogens, but which stimulate the pituitary (lactones); it increases the amount of estrogens excreted in the urine. In addition, many workers have shown that the liver destroys estrogens; Segaloff (1947) found that progesterone would oppose this inactivation by the liver, but this needs to be confirmed. Several workers have stated that estrogen acts on the formation and on the maintenance of the corpus luteum; only its maintenance is discussed here. As early as 1930, Goormaghtigh and Amerlinck declared that estrogen prolongs the duration of the corpora lutea. Bibliographical references can be found in my book (1945a); in particular, Westman, Robson, Allen and Heckel, Klein and Mayer should be cited.[15]

Höhn and Robson (1949) have been interested quite recently in this problem, and have implanted estrogen crystals in the corpus luteum of the hypophysectomized rabbit. Mayer succeeded in making such an implantation in 1948. It is certain that estrogen is able to prolong the activity of the corpus luteum of the rabbit,[16] the rat, and woman, and the complexity of the endocrinal equilibrium is apparent. Thus estrogen is capable of prolonging the activity of the corpus luteum, it can reinforce the action of the hormone of the corpus luteum on receptors, but it can also, in certain doses, neutralize this hormonal action.

It becomes, therefore, of great interest to discover whether estrogen acts on the excretion of pregnanediol in the urine. This question is still under discussion. Pattee *et al.* (1940), found that the injection of estradiol into women in the luteal phase diminished the pregnandiol in the urine. On the other hand, Smith *et al.* (1946), found that the injection of diethylstilbestrol in pregnant women (toxemic pregnancy) increased the pregnanediol. But Davis and Fugo (1947, 1948) did not find this increase, and recently Sommerville *et al.* (1949) stated that diethylstilbestrol caused a decrease of pregnanediol in the urine in both normal and diabetic pregnant women. These, then, are contradictory results.

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[14] These derivatives pass into the urine; they can be reactivated by the hydrolysis of urine with HCl in the presence of zinc (Smith).

[15] See the recent work of Mayer and Klein (1948).

[16] Robson has shown that progesterone does not act in opposition to estradiol in the maintenance of the corpus luteum in the hypophysectomized rabbit (Robson, 1939).

If estrogen maintains the activity of the corpus luteum, it would seem extraordinary that it should cause a decrease in the amount of pregnandiol; of course estrogen might be acting not only on the activity of the corpus luteum, but also on the metabolism of progesterone, or on the passage of pregnandiol through the kidney.

VIII. Related Interactions

The correlations that form the object of this review go beyond the compass of relations between natural estrogens and progesterone. Some work done with diethylstilbestrol has already been mentioned. Certainly artificial estrogens are capable of acting like natural hormones, and of entering into functional relations with progesterone; it seems then, that physiological activity may be more important than chemical structure in this connection.

Parkes *et al.* (1938) investigated the action of ethinylestradiol and of diethylstilbestrol on the pregnancy of the rabbit. These two substances, administered by mouth, "inhibit" the effect of progesterone. They oppose formation of the endometrial lace, prevent ovo-implantation, and interrupt developing pregnancy. According to these workers, the effects are the same as those which the writer found with natural estrogens; but the quantities of diethylstilbestrol necessary to neutralize progesterone appear to be very high.

Robson (1938b) studied triphenylethylene, and showed that this artificial estrogen neutralizes the specific effects of progesterone on the endometrium and the myometrium of the rabbit, exactly as does a natural estrogen. Werthessen and Gargill (1945) castrated rabbits 24 hours after copulation, and gave them progesterone together with diethylstilbestrol; the latter prevented formation of the endometrial lace induced by the progesterone, but would not exert a preventive action on the growth of the free blastocyst. However, the experimental tables of the authors are difficult to understand.

It must also be mentioned that some substances different from progesterone are also capable of opposing the action of estrogen under certain conditions.

It is known that desoxycorticosterone can exercise an action like that of progesterone; it opposes the keratinizing action of estrogen on the vagina (del Castillo and di Paola, 1940). Conversely, estrogen opposes the progesterone-like action of the desoxycorticosterone on the uterus (Kehl, 1941).

Testosterone also prevents the keratinizing action of estrogen on the vaginal epithelium (Courrier and Cohen-Solal, 1937b; Robson, 1936a, 1937, 1938c). In this antagonism, testosterone appears to be more effective than progesterone; and when the propionate is used, it is possible to account for the persistence of its action. Following injections of testosterone propionate in the castrated female rat, the vagina maintains insensitivity to estradiol for several weeks.

It is interesting to consider the polyvalent steroids, that is to say, those which clearly possess several functions. Pregneninolone, or ethinyltestosterone, is a remarkable substance, for it is like progesterone, androgen, and estrogen all at the same time. While these three functions are carried by different molecules, there are definite relations between them. Progesterone and testosterone can oppose the action of estrogen on vaginal keratinization, and mucification results from this hormonal conflict. Moreover, estrogen can oppose the action of progesterone on the uterus of the rabbit, for example. What takes place between the three functions when they are carried by the same molecule?

Courrier and Jost (1939b) have shown that pregneninolone is capable of maintaining pregnancy in the castrated rabbit; a fact which leads to the supposition that there is synergism between its estrogenic and progestational functions, and in this case the quantity of estrogenic function must be slight in relation to the progestational function. Emmens and Parkes (1939), however, stated that pregneninolone induced vaginal keratinization in the castrated rat. In this case, then, the estrogenic function must dominate over the functional progesterone and androgen.

Actually, Courrier *et al.* (1943) have noted that the action of pregneninolone on the vaginal epithelium is characteristic, and is by no means that of a true estrogen; the keratinization lasts but a short time, and is soon replaced by mucification; the action is similar to that produced by a mixture of estrogen and progesterone in which progesterone is dominant. The estrogenic function of pregneninolone must therefore be weak, and it is understandable that the substance can maintain pregnancy in the castrated rabbit.

This estrogenic function is widespread; it seems also to exist side by side with the androgenic function in dehydroandrosterone and testosterone. It is possible that progesterone and desoxycorticosterone possess this function also, but weakly (see above).

This review, in some respects too long and in others too short, has proven, I believe, the existence of functional relationships between hormones like estrogen and those similar to progesterone. Regardless of what name is given to these interactions, they constitute an interesting problem which should have considerable influence on clinical practice.

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The Physiological Actions of the Hormones of the Posterior Lobe of the Pituitary Gland

Part 2.—Actions Other Than Those upon the Circulation and the Secretion of Urine

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VI. INTRODUCTION

In *Vitamins and Hormones*, Vol. VII, the actions of the hormones of the posterior lobe of the pituitary gland upon the circulation and upon the secretion of urine were reviewed. In what follows the remaining actions will be treated similarly. While knowledge concerning the actions already reviewed is far from complete it is on the whole more fragmentary in the case of those now to be discussed. The careful reader will not be satisfied with a number of things. For example, the divergent results concerning the action of the pressor hormone upon the intestines are puzzling, the action of the oxytocic hormone upon the intestines requires more study, confusion is appearing in connection with the action of the pressor hormone upon the uterus, and the function of the melanophore hormone in mammals, if it has any, remains to be discovered. It is only possible to set down the states of affairs as they exist in many cases, ordering the data when possible, but leaving things open when nothing but more facts can bring clarity.

VII. Effect upon Respiration

Pankow (1912) was the first to record the effect of pituitary extract upon respiration. In the rabbit he observed a short period of apnea which occurred immediately after the injection and then a second period somewhat longer lasting than the first. He was of the opinion that the first period of apnea was of peripheral origin since it could be abolished with atropine or by section of the vagi; the second period of apnea he could not explain but suggested that it was due to central action or to bronchial constriction.

Pankow's observations have been amply confirmed but variations which he did not observe have been noted also. Subsequent investigators (Führer (1913a) and Roberts (1923)) have not found the first effect to be abolished by atropine or vagotomy though it may be less marked. Their data and those of Schoen (1928) also show not only two periods of apnea but sometimes respiratory effects which look like short periods of Cheyne-Stokes breathing.

Instead of seeking separate causes for Pankow's first and second apneic periods, therefore, it would seem simpler to regard pituitary extract as causing a Cheyne-Stokes type of respiration, of varying duration. Gollwitzer-Meier (1926) has shown that if the extract is injected slowly the typical effect of Pankow is not seen; instead there is a simple decrease in the amplitude of the respiratory movements.

The cause of the respiratory action may be a consequence of the circulatory action, a view supported by one of Führer's tracings which shows the absence of the respiratory as well as of the vascular effect when a second injection was made soon after the first.

In cats pituitary extract does not produce the characteristic effect it produces in rabbits. Führer observed no period of apnea. The slight effect shown in one of his tracings is most obviously explained as a consequence of the circulatory disturbance. Nice *et al.* (1914) observed a slight diminution in the respiratory rate; Roberts (1923) states that a diminution in the depth of respiration occurs.

The respiratory action of pituitary extract in the dog has not been studied much. It is clear from the work of Jackson and Mills (1919–20) that the extract, if free from histamine, has no bronchoconstrictor action even though doses large enough to cause profound vasoconstriction are employed. Roca's (1921) data lead to the same conclusion. Kolls and Geiling (1925) state that periodic panting occurs in normal but not in etherized dogs. According to Heymans and Heymans (1926) a decrease in the depth of respiration but not in rate is produced in dogs by pituitary extract. This effect appears to be due to a vagal reflex. According to Wittich (1943) pitressin prevents histamine shock in guinea pigs.

VIII. EFFECT UPON THE MOVEMENTS OF THE ALIMENTARY TRACT

1. Introduction

Fodera and Pittau (1909) seem to have been the first to notice the action of pituitary upon the alimentary tract—repeated defecation followed by tenesmus in dogs. Bell and Hick (1909) almost simultaneously, in the course of some experiments on the action of various tissue extracts upon the uterus of the rabbit, noticed that defecation occurred. A blood pressure tracing also showed several slight rises superimposed upon the main curve which they state were "brought about, no doubt, by the effect of pituitary extract on the intestinal muscles." They thereupon proposed the use of pituitary extract in paralytic distension of the bowel and shortly thereafter Bell (1909) reported several cases showing a useful clinical effect upon the bowel postoperatively. Since then much experimental and clinical work has been done, but, in spite of this, considerable confusion still exists in the literature of the action of pituitary extract upon the alimentary tract. Efforts have, for the most part, concerned the establishment of simple facts and little attention has been given to questions of mechanism.

2. The Active Substances

In all probability the substances responsible for the actions of pituitary extract upon the alimentary tract are the pressor and oxytocic hormones. As will be seen in what follows several investigators have doubted this but the present status of the question leaves little doubt that the statement is true. Preparations of the pituitary hormones may now be purified to such an extent that their actions cannot be explained in terms of contaminants. Histamine, which has probably been in the minds of some who have doubted the participation of the pressor and oxytocic hormones in the effect of the extract, is active in very small amounts upon the alimentary tract and was probably a factor in much of the published work, but preparations may be made now which are histamine-free and also very potent. In a paper of Melville and Stehle (1934b) for example, the comparative effects of 0.02 mg. of a histamine-free pressor preparation (50 times as potent as standard pituitary powder) and of 0.01 mg. of histamine dihydrochloride are shown. The results imply that the former has about the same degree of activity as the latter upon the isolated lower colon. The hormone concentration in this experiment was 1:5,000,000. Since the guinea pig uterus is frequently stimulated by the oxytocic hormone in a concentration of 1:500,000,000 it is obvious that the intestine is only a hundredth as sensitive to the pressor hormone as the uterus is to the oxytocic.

The only implication that the melanophore hormone may have an action upon the gut is in a paper by Kaufmann (1927) in which it is stated that extracts of the pars intermedia and pars neuralis, which had very different oxytocic potencies, had equal actions upon the isolated intestine of the rabbit. However, melanophore preparations seem not to have been subjected to a direct test.

3. The Mechanism of Action

So far as it is possible to draw any conclusions it may be stated that the hormones of pituitary extract exert their effects upon the alimentary tract by direct action upon the contractile tissue. The argument for such a mechanism is better in the case of the uterus than in the case of the intestines. There is no reason to believe that the autonomic nervous system is involved. The response of the isolated rabbit intestine, which the pressor hormone certainly excites, rules out central and sympathomimetic actions. The opposite effects upon the blood pressure produced by the pressor hormone and acetyl choline eliminates a parasympathomimetic action; no one seems to have tried the action of pituitary extract upon the rabbit intestine after atropine. In the intact animal the intense constriction of the splanchnic blood vessels caused by the pressor hormone makes one think of the possibility of an indirect action. Moreover, there seems to be a distinct lag in the action, the pressor effect preceding the intestinal contraction by a minute or so; while suggestive. this fact alone permits no sure conclusion.

4. Action in Rabbits

a. Isolated Intestine and Stomach. Much of the early work done on the isolated intestine of the rabbit cannot be interpreted. Many of the extracts used were probably almost or wholly devoid of the pituitary hormones and in some cases whatever activities they possessed were probably due to nonhormonic constituents. Histamine may have been responsible for some of the actions recorded. Other extracts were probably so acid that the addition of them to the bath changed the reaction to such an extent that this, rather than the hormones, determined the result. The importance of the pH factor was not realized until Gruber (1926) investigated it. The portion of the intestine used was also often disregarded.

Bayer and Peter (1911) and Guggenheim (1914) doubtless used extracts contaminated with histamine. They found their materials to excite intestinal activity. The alcohol-soluble portion of Bayer and Peter's preparation caused excitation while the residue caused relaxation. In the case of Guggenheim's preparation alkali did not destroy the substance responsible for the excitation. Histamine was probably a factor in Young's (1915) experiments in which he observed some excitation, Walle Shamoff's (1915–16) results (inhibition) were probably due to hormone-free material in some cases and possibly to high acidity in other cases. Alvarez's (1918) failure to observe any effect from pituitrin was also due possibly to disregard of the acidity of the preparation.

The first experiments on the rabbit intestine with what later came to be known as standard pituitary powder were those of Gruber (1926). When the acetic acid extract of this powder was neutralized before use, it was found to increase the amplitude of the contractions and the tone of strips from the duodenum and ileum. Neutralized commercial preparations had a similar effect. At times the excitation was preceded by a momentary inhibition. Kaufmann (1927) performed similar experiments a little later and showed that the ileum was more sensitive than the jejunum. Because heating the extract at 100°C., in 0.2 *N* sodium hydroxide for 5 minutes did not abolish the action upon the ileum she concluded that the active constituent was not the pressor or the oxytocic hormone. Actually she did not test the solution used for the oxytocic hormone but assumed its destruction (and that of the pressor hormone too) from other experiments in which she used 1 *N* sodium hydroxide. More significance attaches to those of her experiments in which she found extracts of the neural and intermediate lobes to exert equal actions upon the ileum although the neural lobe extract had 10–20 times as much oxytocic action as the intermediate lobe.

Kaufmann found the colon to be more sensitive than the ileum to pituitary extract, but in this case the period of excitation was preceded by well marked inhibition. High concentrations (1 to 10,000) she found to cause pure inhibition.

When the separated oxytocic and pressor hormones became available Gaddum (1928) tested these upon intestinal segments taken from different levels. His results with pitressin were very much like those of Kaufmann with whole posterior lobe extract; the oxytocic hormone had no action. Though he made no exact comparison Gaddum thought equipressor doses of pituitrin and pitressin to have equal actions upon the intestine. The German oxytocic and pressor preparations, orasthin and tonephin, were tested by Isaak and Siegel (1929), upon the colon; tonephin caused excitation and orasthin had no action. Their tracing shows no evidence of the inhibitory phase noted by Kaufmann. Elmer *et al.* (1930) have published results which they think indicate that the oxytocic hormone neutralizes the action of the pressor hormone; they used the ileum and the colon.

A peculiar effect of the pressor hormone upon the large intestine has been described by Melville and Stehle (1936); they noted inhibition of segments taken just below the ileocolic sphincter and excitation of segments from lower levels. They state that the oxytocic hormone has no action upon the colon.

It appears reasonable to conclude from the foregoing that pituitary extract excites all portions of the isolated intestine of the rabbit with the exception of the proximal colon which it relaxes, that the effect increases in intensity from the duodenum on, and that the action is due to the pressor hormone.

The only study of the action of pituitary hormones upon stomach strips from the rabbit is that of Robson (1931). He found that both hormones relaxed strips from the lateral fundus and caused contraction of strips from the medial-anterior fundus. The antrum was excited by pitressin and was unaffected by pitocin.

b. Intact, Unanesthetized Rabbit. Franchini (1910a, b), using huge doses of a probably weak extract of the whole pituitary gland, observed diarrhea. He then studied the action of anterior and posterior lobe extracts and decided that the active substance was contained in the posterior lobe. Katsch (1913) with an abdominal window technique, could observe pituitrin and infundin to cause excitation in the small and large intestines and in the cecum and also defecation. Similar results were obtained by Zondek (1920) who observed the cecal action particularly.

c. Anesthetized Rabbit. Isaak and Siegel (1929) have demonstrated clearly the excitatory action of 0.5 units of tonephin upon the duodenum and colon, the effect upon the former being less than that upon the latter. Elmer *et al.* (1930) observed the colonic effect with pitressin. Upon the injection of 0.5 unit of pitressin Melville and Stehle (1934) found the ileum to be more excited than the duodenum, while pitocin was without action. With 0.25 units of postlobin-V (pressor hormone) they found the ileum to be excited but not the duodenum, and postlobin-O (oxytocic hormone) to be without action. They also found the lower colon but not the upper colon to be excited by postlobin-V and neither portion to be affected by postlobin-O. The stomach is first inhibited and then excited by pituitrin (Schoendube and Kalk (1926)).

5. Action in Cats

Young (1915), MacDonald (1925), and Kaufmann (1927) who studied the action of pituitary extract upon isolated segments of the intestine of the cat observed excitation. MacDonald expressed the opinion that the effect was due to some substance not specific to the posterior lobe, possibly the blood pressure depressor substance, which was present in the early pituitary extracts. Gaddum (1928) states that he confirmed MacDonald's results. The cat intestine is rather sensitive to histamine and this compound may have been partly responsible for the excitation. Kaufmann agreed with MacDonald but was less specific in her guess regarding the active agent. Her experiments eliminated histamine, at least as far as her own work was concerned; the effect was much reduced by treatment of the extract with alkali. Moreover, according to Mackay's (1930) work, it would have required about a milligram of histamine to produce approximately the effect which Kaufmann obtained, but the latter used only 1 mg. of glandular material and this, as stated, lost its oxytocic activity on treatment with alkali.

Only two investigators have examined the effect of intravenous injections of pituitary extract upon the cat intestine *in situ*. Dixon (1923) observed excitation of the small intestine and excitation followed by inhibition in the large; he did not give the dosage. Steggerda *et al.* (1938) have recently studied the effect of pitressin and pitocin upon the colon by a Roentgen-ray technique and have shown clearly that three units of pitressin caused contraction; defecation occurred also. Pitocin was without action.

6. *Action in Dogs*

The action of pituitary extract upon the alimentary tract of the dog is in a great state of confusion. Different techniques have lead to results that at present appear to be contradictory.

One of the most constant effects of an intravenous injection of 10 units of the pressor hormone into a normal unanesthetized dog, turned loose immediately after the injection, is defecation and tenesmus. Vomiting also frequently occurs. The oxytocic hormone causes defecation less frequently but often enough to regard it as an action of the hormone. In spite of these facts studies upon isolated segments, loops *in situ*, and Thirty-Vella loops have not provided clear evidence to indicate that such effects should be produced.

a. Isolated Segments. Dale (1909) found an isolated loop to contract slightly; he stated that the sensitivity was small. Gruber and Pipkin (1930) observed excitation in some cases and inhibition in others even though the segments were from the ileum and the dosages the same. They thought that the initial tone determined the effect.

b. Intestine in situ *under Anesthesia.* Dale (1909) states that pituitary extract caused distinct inhibition of the intestinal movements in the anesthetized dog (direct observations). Hoskins (1916) found segments *in situ* to relax after the intravenous injection of pituitrin. Dixon (1923), on the other hand, states that loops of small intestine *in situ* are constricted while loops of colon are momentarily constricted then relaxed.

Melville and Stehle (1934a) could observe no action upon the duodenum or ileum when one unit of the pressor hormone was injected intravenously, but brief relaxation followed the injection of one unit of the oxytocic hormone. Upon the colon also they found the oxytocic hormone to cause inhibition.

c. Fistulas. The experiments of Gruber and Robinson (1930) and of Gruber *et al.* (1931) in unanesthetized dogs with Thirty-Vella fistulas demonstrate a complete loss of tone for a time and then groups of contractions in which the amplitude does not exceed that of the prepituitary control. Whole posterior lobe extract, vasopressin and oxytocin had the same action. On the other hand Carlson (1930) states that pituitary extract and pitressin sometimes had no effect and in other cases caused excitation; decreased activity and relaxation was not observed by him in the unanesthetized dog. Mordwinkin (1928) studied the action of pituitrin upon the colon in a dog provided with a fistula of the cecum and found it to cause relaxation. Quigley and Barnes (1930) observed decreased tone and motility of the small intestine and colon.

The intraintestinal pressure in the obstructed small bowel decreased following the intravenous injection of pituitary extract, vasopressin and oxytocin according to McIntosh and Owings (1928).

d. Roentgen-Ray Experiments. The Roentgen-ray experiments of Melville and Stehle (1934a) leave no doubt that the pressor hormone had definite excitatory actions upon the small and large intestines. The small intestines shorten considerably and the margin becomes serrated. The action of the oxytocic hormone is definite but its significance is not clear; the small intestine assumes the appearance of a broad ribbon, and appears to be shortened but its contents were not found to be passed on to the colon.

e. Effects Upon the Stomach. Galan (1921) states that isolated strips of the stomach are excited by pituitary extract but are relaxed by high concentrations of chloretone-containing preparations. In the anesthetized dog the intravenous injection of the extract caused slight inhibition followed by considerable excitation. Ginsburg and Tumpowski (1918) and Quigley and Barnes (1930) found that pituitrin, vasopressin, and oxytocin decreased the tone of the stomach in dogs provided with gastric fistulas. Rossiisky *et al.* (1936) have found pituitary extract, given subcutaneously, to excite gastric motility in Pavlov pouch dogs.

7. *Action in Guinea Pigs*

Garry (1927) states that the intestine studied by means of the Trendelenburg method is occasionally excited when the concentration is that represented by one part of fresh lobe per hundred thousand and is inhibited by higher concentrations. Simon (1933) found the ileum in a 10 ml. bath to be excited by 0.01 unit of vasopressin. Galen (1921) states that the isolated stomach is excited by chloretone-free extracts.

8. *Action in Man*

The data of those who have used the balloon technique testify without exception to the excitatory action of pituitary extract upon the human alimentary tract. Ginsburg *et al.* (1916) found 1 ml. injected subcutaneously to cause gastric contractions. Carlson (1930) in the case of two ileostomies and two colostomies noted increased peristalsis of the ileum and of the colon; in the former case a period of quiescence preceded excitation and defecation. Bargen and Guthrie (1936) state that contractions were produced in the terminal ileum, and in the transverse and sigmoid sections of the colon. Macdonald and Settle (1936) studied the action of pitressin and pitocin in fifteen patients with colostomies and found that 1 to 2 units of pitressin given intravenously invariably caused stimulation whereas 5 to 10 units of pitocin caused stimulation in only three out of eleven trials. Five units of pitocin did not prevent the action of 1 unit of pitressin.

Roentgen-ray studies of the action of pituitary extract have been made by Pancoast and Hopkins (1917), Schoendube and Kalk (1926), Gorke and Deloch (1922), and Rondelli (1929). According to the first-named, in the stomach primary inhibition was followed by increased peristalsis but the small and large intestines were hardly affected. Schoendube and Kalk's results on the stomach were similar to those of Pancoast and Hopkins. Gorke and Deloch observed increased peristalsis and tone in the pyloric region, delayed emptying and defecation. Rondelli found the principal effect (excitation) to be in the colon; defecation sometimes occurred.

IX. ACTION UPON THE GASTRIC MUCOSA

In 1910 Franchini, in a study of the actions of pituitary extract, gave colossal doses intravenously, subcutaneously and orally to rabbits. Besides repeated defecation and diarrhea, ulcerations, and hemorrhages in the stomach and intestines were frequently noted. Anterior lobe extracts had little or no effect but posterior lobe extracts produced the same results as those of the whole gland.

Many years later this action was rediscovered by Dodds and his collaborators (1934, 1935, 1937). Usually they employed a posterior-lobe preparation of their own (75 mg. per kg. in rabbits, guinea pigs, rats and mice) but ordinary pituitary extract and a pressor fraction which they made according to the procedure of Kamm *et al.* (1928) caused ulceration also. Their papers give the impression that they thought the posterior lobe to contain a separate gastrotoxic factor since they state that the effective agent had been removed from the oxytocic but not from the pressor preparation of Kamm and his associates and also that the pressor and oxytocic substances of whole posterior lobe extract have no relation to the phenomena described. Bergami (1935), Nedzel (1936) and Hanke (1936) have confirmed the findings of Dodds and his associates.

Selye *et al.* (1936) were able to reproduce the results of Dodds and his collaborators with 1 mg. (200 units) of the pressor preparation of Stehle and Fraser; the oxytocic preparation and the melanophore preparation of Stehle were without action upon the gastric mucosa. There is little doubt, therefore, that the gastric ulcers are a toxic manifestation of large doses of the pressor hormone.

X. EFFECTS UPON GLANDULAR SECRETION

1. Salivary Secretion

In the cat, pituitary extract does not affect the secretion of saliva, according to Frankl-Hochwart and Fröhlich (1910). In dogs it causes a decrease (Solem and Lommen (1915)) and even diminishes the effect of pilocarpine (Ishunina (1927)). A decrease was noted in man by Gorke and Deloch (1922).

2. Gastric Secretion

The results of the early work of Rogers *et al.* (1916) upon gastric secretion in Pavlov pouch dogs may be dismissed because their extracts had only a depressor action upon the blood pressure. Alpern (1923), following the subcutaneous injection of an extract of the intermediate lobe, found the rate of secretion and the acidity, during sham feeding, to be diminished and neither returned to normal levels until the fourth day after the experiment; the extract also prevented the normal secretory response of pilocarpine. Essentially the same results were obtained by Elkeles (1926) with hypophysin though the effect was not so lasting; the normal secretory effect of histamine was not affected. Dionesov (1931) also found the rate of secretion during sham feeding to be diminished by pituitrin.

Studies of the effect of pituitary extract on the rate of gastric secretion and acidity in man have been published by Pal (1916), Hess and

Gundlach (1920), Schoendube and Kalk (1926), Hoffman (1921), Gorke and Deloch (1922), Cascao de Anciaes (1926), and Hottmann (1936). The concentration and absolute amount of acid is diminished according to most, but not all, workers. In the fasting stomach, according to Schoendube and Kalk, the volume of secretion is diminished, too, but following a test meal Hoffman and Gorke and Deloch found the secretion to be increased. Vilardell (1932) could not confirm the results of Drouet and Simonin (1932) who had reported that pituitary extract suppressed the secretion of hydrochloric acid produced by the administration of histamine.

3. Pancreatic Secretion

Pemberton and Sweet (1908) and Edmunds (1909) found that in dogs the pancreatic secretion stimulated by secretin was diminished by pituitary extract. Schaefer and Herring (1908) state that the normal rate of secretion is not increased. Eidinova (1938) concluded that the effect depends upon the initial state of the secretory cells.

4. Secretion of Bile

Adlersberg and Noothoven van Goor (1928) and Kalk and Branisteanu (1932) have reported a diminution in the secretion of bile in the rabbit and in the dog respectively following the injection of pituitary extract.

The gall bladder does not seem to be contracted by pituitary extract. Brugsch and Horsters (1926) and Erbsen and Damin (1927) have observed relaxation of isolated strips from the gall bladder of guinea pigs, and the latter of similar strips from the dog as well. Adlersberg and Noothoven van Goor could observe no contraction of the gall bladder of the rabbit.

5. Secretion of Milk

In 1908 Ott and Scott (1910–11) noted that when the mammary gland of the goat was cannulated the intravenous injection of pituitary extract caused a marked increase in the flow of milk for 5 minutes. This effect was soon confirmed by Schaefer and Mackenzie (1912), while Mackenzie (1911) and Schaefer (1913) also observed it in a human subject. Determinations of the total daily output of milk in the cow and goat during normal days and days when the extract was given have shown that the total daily quantity is not affected (Gavin (1913), Hammond (1913), Hill and Simpson (1913–14), Turner and Slaughter (1930), Rothlin *et al.* (1922)). The conclusion has usually been drawn that the secretion of milk as such is not effected but that the contents of the gland are "pressed out," perhaps prematurely. This idea gains credence from the fact that the milk produced under the influence of pituitary extract is characterized by an abnormally high fat content.

In the experimental data of this subject there is nothing to indicate that the action is of physiological significance. Large doses have always been employed in producing the effect. On the other hand a physiological function is not impossible; it may be that one of the posterior lobe hormones plays a role in maintaining the physical flow of milk even though it does not affect its actual secretion.

No one seems to have determined which of the posterior lobe hormones is responsible for the action. In the course of some experiments on the action of pituitary extract upon the secretion of urine the reviewer once prepared a bladder fistula in a dog not known at the time to be pregnant. In using this animal 18 days after parturition a slight flow of milk occurred immediately following the injection of 1 unit of a pressor preparation.

XI. Action upon the Uterus

1. Introduction

The first evidence showing that an extract of the posterior lobe of the pituitary gland causes the uterus to contract is to be found in a paper by Dale (1906). He wrote: "The pressor principle of the pituitary (infundibular portion) acts on some constituent of the plain muscle fibre other than that which is excited by adrenaline." The recorded contraction of the uterus of the cat was probably due in part to histamine (the type of the blood pressure indicates histamine contamination) but since the record shows that the extract produced a good pressor effect it is beyond question that the extract was active in an oxytocic sense apart from its histamine content. Three years later Dale (1909) made a more extensive study of pituitary extract; in it his earlier observation was confirmed. He used uteri from the dog, rabbit, guinea pig, and rat. About the same time, Ott and Scott (1908) and Bell and Hick (1909) were investigating various glandular and tissue extracts for oxytocic activity but it is impossible to evaluate their data satisfactorily. Ott and Scott say that they obtained better effects from extracts of the mammary and prostate glands than they did from pituitary extract, and Bell and Hick obtained their best effects from a uterine extract. Thus it is likely that these workers were observing the action of histamine rather than of specific substances in the extracts used. This statement does not apply to the pituitary extract employed by Bell and Hick, for it was an extract furnished them by Dale and was the same preparation employed by Dale in his own experiments; however, as stated, Bell and Hick considered it inferior to their uterine extract.

2. The Oxytocic Hormone

The oxytocic hormone appears to be a polypeptide containing arginine(?), tyrosine, cystine, proline, perhaps leucine and, in all probability, other as yet unidentified amino acids. Crude extracts of the posterior lobe contain varying amounts of histamine, which was at one time believed by Abel and Kubota (1919) to be the oxytocic constituent of the pituitary.

The oxytocic hormone, like the pressor hormone, elicits effects when very small quantities are employed. In the rabbit at the end of gestation, according to Robson (1933a), as little as 0.001 unit (or in terms of potent oxytocic preparations 0.004 μg.) is detectable by its uterine action. The common postpartum dose in the human, 10 units, is only 0.04 mg. In the assay of pituitary extract using the isolated guinea pig uterus and a 100 ml. bath, about 0.05 unit produces a suitable contraction; the concentration under these conditions is 1 part in 500 million.

Preparations of the pressor hormone of pituitary extract have never been obtained free from oxytocic activity, the best preparations having 4 or 5 oxytocic units for each 100 pressor units. This means that either the pressor hormone has some oxytocic action or that the methods thus far used to separate the two hormones are at fault. If the second explanation is true it is remarkable that the very different methods of separation which have been used by Kamm and his collaborators (1928) and by Stehle and Fraser (1935) should result in preparations contaminated to about the same extent. In this connection it is of interest to note that several investigators have observed that the pressor hormone has an action upon the human uterus. Robson (1933b) found that uterine strips taken early in gestation reacted to vasopressin but not to oxytocin.

Upon the normal human uterus *in situ* Moir (1934) observed that pitressin was more active than oxytocin; the action was slow in developing. In dysmenorrhea Woodbury *et al.* (1947) found that 1 unit of pitressin caused greater uterine activity than did 1 unit of pitocin.

The response of the isolated uterus of various species to the oxytocic hormone is augmented by magnesium ions (Genell, 1938). When magnesium chloride is present to the extent of 0.005% the sensitivity of the uterus is five times as great as when magnesium is absent. Fraser (1939) has reported similar results and has also shown that the pressor hormone, in the presence of magnesium salts, exerts a strong oxytocic action and becomes equal in potency to the oxytocic hormone when 0.1% of magnesium chloride is present in the bath.

Entirely apart from the excitatory action of pituitary extract upon the uterus a few references are to be found in which a depressant action is described. Attention will be called to these in what follows.

3. *Mechanism of Action*

The oxytocic hormone seems to excite the uterine musculature directly in all species, elasmobranchs excepted (Dreyer, 1946). The autonomic nervous mechanism is not involved. After chrysotoxine in the cat (Dale, 1906), when adrenaline causes relaxation, pituitary extract causes contraction and, in the nonpregnant guinea pig in which adrenaline causes relaxation of the uterus, pituitary extract causes contraction; these facts eliminate the participation of any sympathetic mechanism. The uterine contractions produced by pituitary extract are not abolished by reasonable doses of atropine (Sugimoto, 1913), hence the parasympathetic mechanism is not involved.

As in the case of the action of the pressor hormone upon the blood vessels, the uterus is refractory for a time to a second application of pituitary extract (Fühner, 1913). According to Weinstein and Friedman (1935) this is due to the pressor hormone, which not only renders the uterus refractory to the oxytocic hormone but even reduces its spontaneous activity. When the pitocin was employed no refractory period was noted in their experiments.

4. *Influence of Other Hormones upon the Effect of the Oxytocic Hormone*

a. Progesterone. In the case of the rabbit, uterine sensitivity varies during pregnancy. Sahako (1925) showed that the isolated uterus of the pregnant animal was insensitive to pituitary extract. This he attributed to the influence of corpora lutea and showed that at the end of gestation, when corpora luteal influence has disappeared, the uterus again responds to pituitary extract. A year later (1926) he found that the uterus *in situ* of the pregnant animal was less sensitive than the uterus of the nonpregnant.

Knaus (1926) discovered that for 18 days after copulation pregnancy cannot be interrupted by even large doses of pituitary extract but that thereafter smaller doses are effective in causing abortion and reach a minimum on the 31st to the 32nd day. Later (Knaus, 1927) he examined isolated sterile uterine horns (produced by ligature of the Fallopian tube on one side) at different stages of pregnancy and found that they showed the variations in sensitivity to pituitary extract to be expected from the earlier work. Robson (1933a) somewhat later tested the isolated uterus at different stages of pregnancy and obtained similar results; according to his data the isolated pregnant rabbit uterus is about as sensitive to the pressor hormone as to the oxytocic. Though the work just cited implied that the corpus luteum was connected with the phenomena described, Knaus (1930) converted this implication into a certainty when he found the sensitivity to pituitary extract of excised uteri from normal, nonpregnant rabbits to be decreased when they had received injections of corpus luteum extract.

For a time there was uncertainty concerning the substance in the corpus luteum responsible for this desensitizing effect but the work of Makepeace *et al.* (1936) proved that pure progesterone injections desensitize the subsequently excised uteri of ovariectomized rabbits, *in vitro*, and the work of Robson (1936) indicated that progesterone desensitizes the uterus *in vivo*. Whether corpus luteum extracts also contain a desensitizing substance different from progesterone, as maintained earlier by Robson and Illingworth (1931–32) and by de Fremery *et al.* (1932), seems to remain an open question. Allen (1937) found that if estrin is given simultaneously with progesterone to ovariectomized rabbits the isolated uterus then not only failed to contract on the addition of pituitrin to the bath but actually relaxed. Relaxation of the uterus *in situ* under similar experimental conditions has been noted by Robson (1936) who attributed it to the pressor hormone; pituitrin and vasopressin produced the effect in question but oxytocin did not.

Prolan injections produce, in immature rabbits, uteri which do not respond to pituitary extract, according to Siegmund (1930c), Winter (1931–32), and Robson (1932–33). The last named observed similar results with preparations of the anterior lobe of the pituitary; he observed that when pitressin was used instead of pitocin the uterus relaxed.

The uteri of species other than the rabbit are not rendered insensitive to pituitary extract by the corpus luteum hormone. According to Siegmund and Kammerhuber (1931) the uterus of the mouse at all stages of the menstrual cycle and of pregnancy is contracted by pituitary extract. This also holds for the rat (Knaus and Clark, 1926, Siegmund, 1930a, b) the cow (Graf and Morgenstern (1929)), the pregnant guinea pig (Sommer (1931)), and the spayed cat (Robson and Schild (1938)).

In the human the data are contradictory. Knaus (1929), found that in this case the uterus responds till the fourteenth day of the menstrual cycle but not after the sixteenth day, when, presumably, the corpus luteum is functioning. Schultze (1931) observed no response early in the cycle but did after the fourteenth day. Moir (1934) found pituitary extract to cause contraction in all stages of the cycle, as did Kurzrok *et al.* (1937) and Henry and Browne (1943). During pregnancy Robson (1933b) found that uterine strips taken at the twelfth to the fourteenth week of gestation did not respond to 2 units of oxytocin, but that after 17 weeks sensitivity increased regularly.

b. Estrogens. In 1928 Bourne and Burn (1928) reported that estrin* sensitized the isolated guinea pig uterus to pituitary extract. Heller and Holtz (1932) and Jeffcoate (1932) repeated this work using crystalline estrone and could observe no potentiating action. Neither could Pompen and Gomperts (1932) using menformon. On repeating their own experiments with crystalline estrone Bourne and Burn (1932) withdrew their original conclusion. One would hardly expect estrone to sensitize the uterus *in vitro* to pituitary extract. Like other sterols it probably produces its effects slowly by causing alterations in the morphological properties of certain tissues which may be paralleled by changes in physiological properties.

While the isolated uterus of the guinea pig is not sensitized to pituitary extract by the direct application of estrogens there is no doubt that the uterus *in situ* of several species is sensitized. Miura (1926) showed that in immature rabbits the injection of a placental extract (which may be assumed to have contained estriol glucuronide) at one to two day intervals for a week or two caused the uterus to increase enormously in size and to become much more sensitive to pituitary extract than that of a littermate.

Parkes (1930) discovered that mice, 13- to 17-days pregnant, aborted immediately when a series of hourly injections of estrin was followed by 1 unit of oxytocin, but in the absence of estrin they did not abort even when 5 units of oxytocin were administered. Similar results were obtained with pure estrone instead of estrin by Robson (1935a) and by Marrian and Newton (1935) with estrone and estriol. The former showed also that the excised uteri of ovariectomized mice which had received estrone had been sensitized to pituitary extract and that the excised uteri of similarly injected mice 12-days pregnant were as sensitive to oxytocin as the uteri of normal mice at parturition.

In rabbits Pompen and Gomperts (1932) and Zeldenrust (1935) found that the uteri *in situ* after injections of menformon (estrone) and dimenformon (estradiol benzoate) respectively were as much as forty times as sensitive to pituitary extract as those of nontreated animals. Later van Bokkuun (1936) obtained similar results with estrone and estradiol.

Robson (1933c) studied the sensitivity of the isolated uterus to oxytocin after a series of injections of crystalline estrone into the ovariectomized rabbit and found the sensitivity greatly increased. This was also true (Robson (1935b)) in the pregnant rabbit after the twenty-first day but before that day the sensitizing action to oxytocin was less certain; in hypophysectomized but otherwise normal rabbits estrin caused sensitization to oxytocin.

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* "Estrin" and not "estrone" is employed here and in several other places because it is the term used by the author or authors cited. In this case it was not pure estrone and this may have been true in some of the other instances also.

In spayed cats Robson and Schild (1938) could observe no sensitizing action of estrone on the uterus *in situ* to oxytocin nor could Knaus and Clark (1926) detect any certain change in the sensitivity of the isolated rat uterus to pituitary extract in estrus, diestrus and pregnancy even though in this species progesterone has no desensitizing action toward the pituitary response.

Estrogens are not essential for the action of pituitary extract upon the uterus. The uteri of ovariectomized guinea pigs (Athias, 1921) and of rabbits (Makepeace *et al.*, 1936) still react.

c. Insulin. According to Joachimoglu and Metz (1925) the action of pituitary extract upon the uterus is inhibited by the simultaneous application of insulin.

XII. ACTION UPON THE CERVIX OF THE UTERUS

Newton (1934) found that the uterine cervix of the guinea pig does not respond to pituitary extract no matter whether it is from animals in estrus, after estrin injections, pregnant or normal. In goats (Newton (1937)) 13- to 20-weeks pregnant, oxytocin caused contraction of the cornua but not of the cervix.

XIII. EFFECT UPON CARBOHYDRATE METABOLISM

1. Blood Sugar Changes

On the basis of theoretical considerations concerning glycosuria in acromegaly Borchardt (1908) tested the action of an extract of the whole pituitary of the horse in rabbits and observed that hyperglycemia and glycosuria occurred. Claude and Baudouin (1912) showed that the active substance was contained in the posterior lobe but not in the anterior lobe. This hyperglycemic action has been amply confirmed and shown to hold for other species also.

Partos and Katz-Klein (1921) and Stern (1925) state that in the rabbit on a green diet, and in infants, respectively, the hydremic action of pituitary extract may conceal the hyperglycemic action. Himwich *et al.* (1930) found the blood sugar to be decreased by pitocin and pitressin in dogs anesthetized with amytal. However, in dogs anesthetized with chloralose Houssay and Di Benedetto (1933) observed hyperglycemia and, in the case of rabbits, Thaddea and Waly (1933) and Haferkorn and Lendle (1933) using tonephin in animals anesthetized with pernocton, and Campbell and Morgan (1933) using pituitary extract in animals anesthetized with nembutal, observed hyperglycemia as in the case of unanesthetized animals.

Following the hyperglycemia several investigators (Dresel, 1914, Myrhman, 1925–26, Blotner and Fitz, 1928, Lindlau, 1928, Velhagen (1929), Thaddea (1933)) report a hypoglycemia. The hyperglycemia is of short duration when the extract is administered intravenously and may be missed entirely if analyses are made too long after the injection. According to Soularie (1944) the period of hypoglycemia is followed by a second period of hyperglycemia equal in intensity to the initial hyperglycemia. Even when repeated injections of pituitary extract were made Velhagen found that hyperglycemia was not maintained but gave way to hypoglycemia.

The glycosuric effect has not received much attention. Borchardt was doubtful about its occurrence in dogs, but Goetsch *et al.* (1911) showed that it occurred if pituitary extract was administered along with quantities of sugar which in themselves caused no glycosuria. Miller and Lewis (1912) could detect glycosuria only once in 16 experiments on dogs, even though they used huge doses of pituitrin. Claude and Baudouin refer to pituitary glycosuria as an alimentary glycosuria, since they could detect it in man only after the ingestion of carbohydrate but not during fasting.

2. *The Hyperglycemic Hormone*

The only conclusion to be drawn at present is that in some species the pressor hormone is responsible for the hyperglycemia and in others the oxytocic.

In man the work of Gavrila and Mihaileanu (1930), Elmer and Scheps (1930), Schroeder (1933) and Thaddea (1933) indicates that the pressor hormone is the hyperglycemic agent; the oxytocic hormone had no effect on the blood sugar. No contradictory results are on record.

In the rabbit it is also the pressor hormone which is responsible for the hyperglycemia effect. Nitescu and Benetato (1930) found that 0.1 cc. of pitressin (presumably 2 units) per kg. raised the blood sugar more than did 0.1 cc. of pitocin (presumably 1 unit) per kg. The pitocin effect in their experiments may have been due solely to the excitement of the injection for Ellsworth (1935) was unable to observe any hyperglycemic action with even 5 units of the oxytocic hormone per kg. when allowance was made for the effect of the injection of saline itself. Ellsworth obtained only a suggestion of a hyperglycemic action with 0.25 unit of the pressor hormone per kg. but 2 units per kg. caused a definite effect. This dose is so large as to throw doubt upon its physiological significance; it may be the secondary result of the circulatory action.

In dogs, according to Holman and Ellsworth (1935), the oxytocic hormone appears to be responsible for the hyperglycemia, the relatively slight action of the pressor hormone corresponding to its content of the unseparated oxytocic substance. The effective quantity of the oxytocic hormone necessary to cause hyperglycemia was small enough, 0.0625 µg. per kg., to justify the opinion that it may be a factor in normal carbohydrate metabolism. An earlier observation of Houssay and Magenta (1929) also indicates that in dogs it is the oxytocic hormone which determines the hyperglycemia. They found that in hypophysectomized animals oxytocin protected against insulin hypoglycemia better than did vasopressin. Recent work of Fraser (1949) makes it clear, however, that the pressor hormone can also cause hyperglycemia in dogs; it is probably the consequence of tissue anoxemia. The hyperglycemic actions of the two hormones do not follow the same time course, that of the pressor hormone being slower in appearing. The results of Himwich *et al.* (1930) throw no light on the active hormone in the dog because of the huge doses they employed (10–30 ml. of pitressin and pitocin).

Velhagen's (1929) experiments, in which he used extracts of the intermediate and neural lobes, indicate that the melanophore hormone has no hyperglycemic action; the intermediate lobe extracts usually had a weaker, and never had a stronger hyperglycemic action, than those of the neural lobe.

3. Mechanism of Hyperglycemia

The conclusion to which work up to the present leads is that pituitary extract hyperglycemia is due to an action of the extract in the liver, which results in the conversion of glycogen to glucose. Clark (1928) could detect no hyperglycemia following pituitary extract if the liver was isolated from the circulation. In decerebrate, eviscerated cats the blood sugar even decreased after the administrations of pituitary extract, indicating that there was no liberation of glucose from the muscles. Geiling and Britton (1927) state that the pituitary extract has no constant effect in the fasting normal animal. In rats starved for 24 hours Lawrence and McCance (1931) could detect only a slight decrease in liver glycogen. Thaddea and Waly (1933) found that when the glycogen stores of rabbits were depleted by administering thyroid the action of tonephin was diminished, and when the liver was poisoned with phosphorus the action of tonephin was absent. Thaddea (1933) could not abolish the action of insulin with pituitary extract in patients with liver disease or Basedow's disease. Imrie (1929) could detect no pituitary hyperglycemia in a depancreatized dog deprived of insulin for 70 hours, the liver then being presumably glycogen free.

The liver glycogen of rats is diminished by pituitrin, according to Burn and Ling (1929) and Gömöri and Marsovsky (1932); the latter also found the muscle glycogen to be unaffected. Thaddea and Waly (1933) analyzed control samples of liver and muscle from rabbits and then injected tonephin. Samples of both tissues taken later showed the liver glycogen to be lowered and the muscle glycogen to be unchanged. Orasthin caused no change in either tissue.

According to most observers pituitary extract does not cause hyperglycemia by stimulating the secretion of adrenaline, for the blood sugar rises after adrenalectomy (Clark (1928), Thaddea and Waly (1933)) and after ergotamine (Clark (1927), Geiling and Britton (1927), Nitescu (1928), Thaddea (1933), Högler and Zell (1935)). Fritz (1928) found that after adrenalectomy pituitary extract caused a decrease in the blood sugar in rabbits and guinea pigs but not in rats. He made his first glucose determinations an hour after the injection and probably missed the hyperglycemic action. While adrenaline probably plays no role in the hyperglycemic action of pituitary extract, actually the secretion of adrenaline is increased by pituitary extract, according to the experiments of Nikolaeff (1929) in which the adrenal glands of cattle were perfused with pituitrin. The action of this endogenous adrenaline would seem to be nullified by pituitary extract as is that of injected adrenaline; this antagonism will be mentioned in a later section.

Sympathectomy does not prevent the hyperglycemic action of pitressin and pitocin in cats according to Bacq and Dworkin (1930).

There is no evidence that pituitary extract causes a decrease in the oxidation of carbohydrate. The respiratory quotient is not significantly diminished (Hines *et al.*, 1927, Bernstein, 1914, Weiss and Reiss, 1923, Zlocgower, 1923) and may even increase concomitantly with the increase of lactic acid in the blood (Weiss and Reiss, 1923, Geiling and De Lawder, 1932).

4. Mechanism of the Late Hypoglycemia

Following the initial hyperglycemia it was stated above that several investigators have found a hypoglycemia to occur. La Barre (1930) has presented evidence that this is due to increased insulin secretion and Velhagen (1929) and La Barre (1930) have shown that pituitary hypoglycemia does not occur after pancreatectomy.

5. The Effect of Pituitary Extract upon Adrenaline Hyperglycemia

Stenström (1913) observed that the hyperglycemic action of adrenaline in rabbits could be almost abolished by large doses of pituitrin. Dresel (1914), Burn (1923), Laurin (1917), Partos and Katz-Klein (1921), and Gurd (1934) have confirmed the observation. Gurd found oxytocin to be less effective than vasopressin, but Silver and Mislowitzer (1931) concluded that pituitrin, orasthin and tonephin caused about the same degree of antagonism. Neufeld and Collip (1939) have reported that an alkaline extract of the posterior lobe, heated at 100°C. for 15 minutes to destroy the pressor and oxytocic hormones, still antagonizes the action of adrenaline. When given alone it had no effect upon the blood sugar level. They conclude that a substance distinct from the known hormones of the posterior lobe is the active agent.

6. The Effect of Pituitary Extract upon Insulin Hypoglycemia

When the blood sugar was lowered by insulin, Burn (1923) observed that it could be raised by pituitary extract. Magenta and Biasotti (1923), Olmstead and Logan (1923), Moehlig and Ainslee (1925), Lawrence and Hewlett (1925), Geiling *et al.* (1927), Velhagen (1929), Houssay and Magenta (1929), Thaddea (1933), and Gurd (1934) have confirmed this result. Of these, the last three used preparations of the separated pressor and oxytocic hormones. Houssay and Magenta found the oxytocic hormone to be more effective in dogs and Thaddea and Gurd found the pressor hormone to be more effective in man and in rabbits respectively. The results are therefore in harmony with what is known concerning the actions of the two hormones in the species investigated. In medullirenal inactivated cats Geiling *et al.* (1929) found the pituitary extract-insulin antagonism to occur as usual. On the other hand, Corkill and Nelson (1947) found that after adrenalectomy the antagonism does not occur. Voegtlin *et al.* (1925) say that they obtained indications that the toxicity of insulin in rats is decreased by subcutaneously, but not intravenously, injected pituitrin. According to some (Wermer and Monguió, 1933, Griffiths, 1941–2, Wislicki, 1943–4) the antagonism between pituitary extract and insulin is not apparent if the insulin is injected intravenously; however, contradictory results are on record (Corkill and Nelson (1947)).

Since there seem to be two opposing factors involved in the action of pituitary extract upon the blood sugar (the hepatic glycogenolytic action and the increased secretion of insulin) the antagonism of the insulin effect evidently depends upon the predominance of the glycogenolytic factor. Several investigators appear to think that the antagonism of the insulin hypoglycemia is not necessarily associated with the hyperglycemic action of the extract. Lawrence and Hewlett (1925) could detect no rise in blood sugar from 1 ml. of pituitrin given subcutaneously in man but nevertheless they found it to abolish the hypoglycemic effect of 15 units of insulin. Geiling *et al.* (1927) and Geiling and Britton (1927) observed that pituitary extract would prevent insulin convulsions without protecting against the hypoglycemia.

7. *The Effect upon Blood Phosphates*

The lowering of the blood phosphates and the diminution in the urinary excretion of phosphates caused by the oxytocic hormone is attributed by Fraser (1949) to diminished insulin secretion which results from the hyperglycemic action, it being known that insulin increases the blood phosphates.

XIV. EFFECT UPON THE OXYGEN CONTENT OF THE BLOOD

It was noticed by Klein and Holzer (1930) that after the injection of pituitrin the venous blood approached arterial blood in its oxygen content. The same was true but to a lesser extent in the case of the carbon dioxide content. The authors attributed the effect to a diminution of capillary permeability. A year later Geiling *et al.* (1931) observed the same action with pitressin and found that pitocin caused changes of a reverse nature. By following the action for a longer period, Geiling and de Lawder (1932a,) found that eventually the oxygen content of venous blood became lower than normal. Geiling *et al.* (1932) could detect no difference between the oxygen dissociation curves of the arterial-like venous blood and the dark venous blood of the subsequent period, and concluded that the high oxygen content of the former was not due to its inability to yield up its oxygen. The possibility of a vascular short circuit being responsible for the effect was dismissed by Klein and Spiegel (1937) because the carbon dioxide difference between venous and arterial blood was not sufficient to merit it. However, Woodbury and Ahlquist (1946) found that pitressin favors the skin circulation at the expense of the circulation in muscle. By assuming that the skin uses less oxygen than muscle they think that the explanation for the arterial quality of venous blood after pituitary extract is evident. The injection of adrenaline prior to the injection of the pitressin prevented the effect, presumably because of the constricting effect of adrenaline upon the vessels of the skin. Experiments upon the oxygen consumption of isolated tissues such as those of Himwich *et al.* (1931) and of Pinkus (1933) have led to nothing illuminating.

XV. EFFECT UPON BASAL METABOLISM

Numerous studies have been made of the gaseous metabolism as affected by pituitary extract (Bernstein, 1914, McKinlay, 1921, Boothby and Rowntree, 1922, Weiss and Reiss, 1923, Odaira, 1925, Nitescu and

Gavrila, 1929, Missal and Johnston, 1914, Lambie and Redhead, 1929, Schill and Fernbach, 1929, Castex and Schteingart, 1930, Himwich and Haynes, 1931, Grollman and Geiling, 1932, Hottmann, 1936). Some have noted no change and others have reported small decreases or small increases. Geiling and De Lawder (1932, b) state that immediately following the injection the oxygen consumption falls and the venous blood becomes arterial-like, then the oxygen consumption rises above the control level and the venous blood becomes more venous than usual. No secondary rise is shown in Odaira's (1925) data for rabbits. The metabolic effects thus appear to be secondary to the decrease in capillary permeability hypothesized by Klein and Spiegel (1937).

XVI. Effect upon the Lactic Acid Content and Reaction of the Blood

A slight decrease of the alkali reserve of the blood and an increase in its hydrogen-ion concentration were noted by Gollwitzer-Meier (1926) to follow the injection of pituitary extract in the rabbit. Draper and Hill (1929) observed a decrease in the alkali reserve with both pitocin and vasopressin. These changes are due directly, in all probability, to the increase of lactic acid in the blood which was reported later by Nitescu and Munteanu (1931), Bischoff and Long (1931–32), Geiling and De Lawder (1932, b) and Himwich *et al.* (1932), which in turn is probably due to impairment of the circulation and the decrease in oxygen utilization, discussed in the immediately preceding section. Collazo *et al.* (1934) regard the lactic acid increase as a toxic effect of the high doses: they found that 5 units of pituitrin in rabbits (methods of administration not stated) caused a decrease in the lactic acid content of the blood.

XVII. Effect upon Chromatophores

A chronological account of the work on the melanophore-dilating action of pituitary extract, in so far as the active substances are concerned, has already been given in the chemical section of this review (Stehle, 1949). There it was concluded that the evidence indicated the existence of two chromatophore hormones in pituitary extract, one which expands the melanophores of amphibians and some fishes and another which expands the erythrophores of some fishes. Jores and Will (1934) claim to have separated the two to some extent but Zondek and Krohn's (1932a) intermedin and Stehle's (1936) melanophore preparation have actions upon both erythrophores and melanophores; whether the ratios of the two activities in intermedin and in Stehle's preparations are the same as in the case of whole posterior lobe extract is not known.

1. Action in Amphibia

The various species of amphibia, frogs, toads (Hogben, 1924), and salamanders (Hogben, 1924, Young, 1935), which have been tested with pituitary extract respond by the expansion of the melanophores. The only exception noted is in the work of Collins and Adolph (1926); they found that pituitary extract caused *Dimyctyles viridescens* (vermilion-spotted newt) to become pale. The response varies considerably with the temperature of the animal (Stutinsky, 1946).

2. Action in Fishes

The reports on the effect of pituitary extract upon the melanophores of fish indicate that the matter is rather complicated. In some species contraction is said to occur, in others there is no effect and in still others expansion results. Intermedin has not been so extensively investigated. It is claimed by Giersberg (1930) that in Phoxinus all of the chromatophores, other than the melanophores, are under hormone control while some of the melanophores are under nerve control. The distribution of nervous and hormonal control is variable in other fishes, however, so that the irregular actions of pituitary extract are comprehensible. According to Brown (1935), in invertebrates, there is a separate hormone which controls the dispersion of each pigment.

Following are the effects of pituitary extract in those species which have been studied.

(a) *Fundulus heteroclitus.* Spaeth (1918) proposed an assay method for pituitary extract based upon the contraction of the melanophores of isolated scales. Wyman (1924) observed contraction of the melanophores to follow the injection of 0.5 cc. of a commercial extract. However, Odiorne (1933) could observe no effect on the melanophores of isolated scales or of scales *in situ* following the injection of pituitary extract, and Foster (1937) could not detect any contraction of the expanded melanophores in the blue-adapted animal after the injection of pituitary extract into the body cavity. Thus in the case of Fundulus the results are contradictory.

(b) *Phoxinus laevis* (Elritze). Abolin (1925) found infundin to cause expansion of both the melanophores and erythrophores. This is confirmed by Giersberg (1930). On the other hand Collin and Drouet (1933) observed pitressin to cause expansion of the erythrophores but not of the melanophores, both in the whole animal and in isolated tissue. Since pitressin is contaminated with the melanophore hormone it would be expected to produce the same qualitative effects as the whole extract. The authors draw the conclusion that the melanophore hormone is different from the erythrophore hormone. Böttger (1936) has proposed the expanding action upon the erythrophores of pieces of fin as the basis of an assay method for intermedin.

(c) *Chrosomus erythrogaster* (red-bellied dace, a near relative of Phoxinus). Whole pituitary extract, pitressin and pitocin cause expansion of the erythrophores and contraction of the melanophores, according to Saphir (1935). This is the only recorded evidence that the oxytocic hormone has any action upon chromatophores. Fisher (1937) observed expansion of the erythrophores in this species with an extract of the posterior lobe of which the neural portion had degenerated because of lesions placed in the hypothalamus.

(d) Pleuronectes (plaice) and Gobius. Meyer (1931) found hypophysin and pituitrin to cause contraction of the melanophores and expansion of the erythrophores.

(e) Perca (perch). According to Giersberg (1930) pituitary extract has no effect on the chromatophores.

(f) Ameiurus (catfish). Darkening occurred following the injection of pituitrin or pitressin (Odiorne, 1933).

(g) Elasmobranchs. Lundstrom and Bard (1932), using the hypophysectomized dog fish (*Mustelis canis*), found that darkening was produced by both pituitrin and pitressin. They found the latter to cause the greater effect, which is incomprehensible if the melanophore hormone is the effective agent; per pressor unit, pitressin is decidedly less active as a melanophore dilator than is pituitrin. Hogben (1936) and Waring (1938) have studied several other genera of elasmobranchs; they employed pitressin treated with alkali to destroy the pressor hormone and observed darkening in light-adapted and hypophysectomized animals.

(h) Other species. Zondek and Krohn (1932a) mention that they have found their intermedin to cause expansion of erythrophores in the stickleback (Gasterosteus), in the bitterling (*Rhodeus amarus*), in several Labyrinthici and in several tropical species. In the case of Rhodeus the melanophores were also expanded, and in the case of the tropical species, *Betta pugnax splendens*, many kinds of chromatophores were expanded. Fleischmann and Kann (1937) tested pituitary extract in several species of Adriatic fish; the melanophores were unaffected but expansion of the other chromatophores occurred.

3. Relation between the Chromatophore Hormones of Vertebrates and Invertebrates

Some invertebrates exhibit color changes similar to those of amphibia and fishes. Since hormones are involved in these changes, the question arises, "Are the chromatophore hormones of vertebrates and invertebrates related?" The chromatophore hormones of invertebrates (Crustacea) have recently been reviewed in detail by Brown (1948).

The hormonal nature of the color changes in invertebrates was first indicated by the work of Koller (1925). He injected blood from a dark-adapted shrimp (*Crangon vulgaris*) into a light animal and observed darkening even though conditions were optimal for light adaptation. Pale animals receiving blood from yellow-adapted animals became yellow, and animals receiving blood from light-adapted animals required 2 days for adaptation to a dark environment whereas normal animals required 1 to 2 hours.

The next step was taken by Perkins (1928). He showed that an extract of the eyes of a white-adapted shrimp (*Palaemonetes vulgaris*) when injected into a dark, blinded shrimp caused the animal to become lighter because of the contraction of the red and yellow chromatophores. This result was confirmed by Koller (1928) in another genus (Leander). He states that the color change was due to "Melaninkontraktion." Koller also found that an extract of the tissues from the rostral region caused darkening in light-adapted animals.

The first trial of a crustacean hormone preparation in a vertebrate was that of Meyer (1931). She injected an extract of the eyestalk of the shrimp (*Crangon vulgaris*) into dark adapted *Gobius minutus* and found the animal to become pale. An extract of the rostral region of *Praunus inermis* caused darkening in light-adapted Gobius. Soon thereafter Perkins and Kropp (1932) found that the eye-stalk extract of *Palaemonetes vulgaris* and *Callinectes sapidus* darkened light-adapted tadpoles. Later (Kropp and Perkins (1933)) observed the same effect in frogs, but in Fundulus only contraction of the melanophores occurred which was limited to the site of the injection. They could not confirm Koller's work showing that extract of the rostral region caused melanophore expansion in the shrimp; they used *Crago boreas* as the test animal. Subsequently Abramowitz (1938) tested an eye-stalk extract from Palaemonetes upon Mustelus, Ameiurus, Rana and Anolis and found the melanophores to be expanded.

The foregoing data are not easy to interpret. The results of Perkins and Kropp and of Abramowitz indicate that the eye-stalk hormone which causes contraction of the melanophores in invertebrates causes expansion of the melanophores in amphibia and non-scaly fishes. The vertebrate and invertebrate melanophore hormones might be identical and these results still be possible, but the experiments of Meyer, with similar eye-stalk extracts which caused contraction of the melanophores of Gobius (melanophores which are expanded by pituitary extract), indicate that the two hormones are not identical. Under these circumstances, one would like to identify the melanophore hormone of the rostral region of crustacea with the melanophore hormone of vertebrates. It expands the melanophores of both phyla according to Meyer (Gobius and Leander) but unfortunately the existence of the rostral hormone, as has been stated, was not confirmed by Perkins and Kropp.

The situation has been further confused by the results of Carlson (1935) showing that the crab Uca which becomes pale on removal of the eye stalks, darkens when an eye-stalk extract is injected. Thus in the crab and in the shrimp eye-stalk extracts produce opposite effects and moreover an eye-stalk extract from shrimps which causes pallor in shrimps causes darkening in crabs. This state of affairs is no worse than that existing in the case of vertebrates, however, for pituitary extract expands the melanophores of Phoxinus and contracts the melanophores of Chrosomus even though the latter species is closely related to the former.

Only a few experiments have been done on the effect of pituitary extract in invertebrates and the results do not support the notion that the melanophore hormones of vertebrates and invertebrates are identical. Perkins (1928) records that the injection of pituitrin in the shrimp (Palaemonetes) causes no change in the chromatophores. Similar results have been recorded by Abramowitz (1937) in Uca (using, apparently, an alkaline extract of the whole pituitary of the sheep).

4. Is There a Melanophore-Contracting Hormone in Pituitary Extract?

Koller's work on the shrimp, cited above, indicates that in invertebrates a hormone may be detected in the blood which contract the melanophores in this crustacean. In reviewing the work on the effect of pituitary extract in fish it was assumed that the contraction of the melanophores, which some investigators have noted, was due to the same substance that in frogs causes expansion of the melanophores. This is not necessarily true, for the phenomenon could also be explained by assuming the presence of a melanophore-contracting hormone.

Meyer's (1931) work is suggestive of this. She found that the blood of a light-adapted flounder (*Pleuronectes flesus*) when injected into a dark-adapted animal caused the latter to become lighter. Hogben and Slome (1931, 1936) also concluded that a substance which causes pallor is secreted by the pars tuberalis of the pituitary in the toad (*Xenopus laevis*). When pitressin was injected into the hypophysectomized animal the darkening lasted longer than in the non-hypophysectomized. Extracts of the pars tuberalis did not cause the hoped for reaction with "consistent success."

XVIII. EFFECT UPON VISION

According to Jores (1933) the melanophore hormone of pituitary extract when placed in the human conjunctival sac decreases the time necessary to adapt the eye to darkness; also in rabbits kept in the dark the melanophore hormone content of the pituitary was greater than in animals kept in the light. Buschke (1928) could not confirm the first mentioned effect. The second results were not confirmed by Koller and Rodewalt (1933) using the frog, but Jores (1935) attributes the failure to the fact that Koller and Rodewald used saline extracts of the hypophyses while he used alkaline extracts. Jores confirmed his own results using mice and Jores and Caesar (1935) found changes in the distribution of pigment in the retina which they believe to support Jores' contention.

Effect upon the Pupil

a. Pupil of the Enucleated Frog's Eye. Pal (1908), Cramer (1908), Borchardt (1908), Franchini (1910b), Kepinow (1912) and Meltzer (1911–12), record that the pupil dilates when placed in a solution of the hormone. The extracts which were employed in the work of these investigators were probably contaminated with histamine which has a mydriatic action but since, according to Gautier (1927), it requires 2 mg. of histamine per ml. to cause mydriasis in the enucleated frog eyes this can hardly account for the effect. Block (1933) in more recent work could detect no effect but her data do not give the concentration employed or indicate that the pH of the solution was controlled. Jores and Caesar (1935) found the former's melanophore preparation to cause dilatation and therefore attribute the effect to the melanophore hormone. It should be noted that failure to undergo spontaneous constriction rather than definite dilatation (1.4%) actually occurred.

b. Pupil of the Mammalian Eye. According to Pollock (1920) when pituitrin was dropped into the conjunctival sac of the rabbit a slight dilatation of the pupil occurred which was not affected by the removal of the superior cervical or of the ciliary ganglion. Subsequent investigators (De Rosa, 1926, Holtz and Jancke, 1936, and Swan and Meyers, 1936) have noted miosis. When the extract was injected subconjunctivally (De Rosa) miosis was followed by mydriasis. Miosis occurred after atropine (Holtz and Jancke, and Swan and Meyers).

After intravenous injection Dale (1909) could detect no effect upon the mammalian pupil. Frankl-Hochwart and Fröhlich (1910) and Githens and Meltzer (1916) state that pituitrin when injected intravenously in rabbits causes miosis. The latter observed the same effect after removal of the superior cervical ganglion. Pollock (1920) found that while constriction is the normal effect dilatation occurs if the superior cervical ganglion is first removed. Steggerda *et al.* (1938) note that the pupil of the cat's eye dilated.

XIX. EFFECT UPON URINARY PIGMENTS

Kabelitz (1941) has reported that the chromatophore hormone increases the amount of urinary pigment excreted in man.

XX. FATE OF THE POSTERIOR LOBE HORMONES

The disappearance of the pressor hormone from the blood after intravenous injection is quite rapid. Jones and Schlapp (1936) found that the plasma of blood samples drawn 5 and 10 minutes after the injection of 20 units of pituitary extract per kg. contained only 50% and 30% respectively as much hormone as blood drawn 0.5 minutes after the injection. Melville (1937) by antidiuretic assay could find less than 5% of injected pressor hormone in the blood of unanesthetized dogs 5 minutes after the intravenous administration of 1 unit per kg., and only a trace 30 minutes after the administration.

Evidence that injected pressor hormone is excreted to some extent by the kidneys was provided by Dale (1909). There was no further contribution to the subject until Larson (1935) studied the problem in more quantitative fashion and found that in anesthetized cats and dogs about 25% of the injected hormone (10 mg. pituitary powder for cats and 8 mg. per kg. for dogs) could be detected in the urine in the hour following the injection, and none thereafter. Similar results were obtained by Jones and Schlapp with decapitate cats.

That fraction of the injected hormone which is not excreted is probably destroyed enzymatically. Heller and Urban (1935) have shown that there is a great difference between the antidiuretic effects of pituitary extracts digested with normal and heated liver tissue. They also observed that, *in vitro*, the antidiuretic substance is adsorbed by various tissues, as shown by the failure of the injected mixtures to give the antidiuretic effect to be expected from the amount of hormone added. Schockaert and Lambillon (1935), about the same time, found that pregnancy serum antagonized the action of pitressin. Since then others have recorded the destruction of the pressor and oxytocic hormones by tissues and by pregnancy blood (Christlieb, 1940, Woodbury *et al.*, 1946). According to Larson (1935) the enzyme involved in the destruction of the pressor hormone is an amino-polypeptidase present in the liver, kidneys, muscle, and blood.

The excretion of the pressor hormone in the urine is of interest in connection with the role of the posterior lobe in the secretion of urine, a theme really outside the limits of this review but not inappropriate for brief mention here.

Presumably when the body is not producing the pressor hormone diabetes insipidus is the consequence. Even the secretion of normal urine therefore involves the action of the hormone to some extent, and it can be detected in normal blood (Melville, 1937). We have seen that injected hormone is in part excreted. In view of these facts it would seem that the pressor hormone should be present in normal urine. Gilman Goodman (1937) thought they could detect it in the urine of dehydrated rats or rats receiving a strong salt solution but not in the urine of hydrated rats or of hypophysectomized, dehydrated rats. Treatment of urine exerting an antidiuretic action with alkali or acid abolished the action. Boylston and Ivy (1938) repeated the experiments and obtained essentially the same results, and Bundschuh and Kuschinsky (1939) found the urine of rats to which saline was given to contain an antidiuretic substance. Arnold (1938), on the other hand, concluded that the antidiuretic substance of urine is not the pressor hormone because in his experiments dialyzed, concentrated urine did not increase the concentration of urinary chloride, and the antidiuretic action was not abolished on subjecting the urine to treatment with alkali. Moreover, the urine of a hypophysectomized dog had as much antidiuretic activity as the urine of a normal dog. Subsequent investigators in this field have also arrived at conflicting conclusions. Walker (1939) was able to confirm the experiments of Gilman and Goodman but on further investigation of the chemical properties of the antidiuretic substance he decided that it was not a product of the pituitary gland—chiefly because of the lability of pituitary extract and the stability of the antidiuretic substance at a pH of 5. Ham and Landis (1942) supported this conslusion by the study of other properties of the two substances. The results of Ingram *et al.* (1939) were in harmony with those of Gilman and Goodman, as were those of Harris (1948), but in both cases they are open to the criticism raised by Landis. However, the hypothesis which is the stimulus for investigations in this field does not fall even if the hormone is proved to be absent from urine. If it is a false hypothesis its falsity will have to be proved by a different kind of evidence.

The fate of the oxytocic hormone has been studied little. Larson (1935) states that following the injection of pituitary extract it is eliminated in the urine of cats and dogs. Whether the experiments were adequately controlled is not obvious. Jones and Schlapp (1936) state that the variable oxytocic activity of normal urine made their attempt to study the problem impracticable. Bundschuh and Kuschinsky (1939) found an oxytocin-like substance in the normal urine of the rat and man. Jones and Schlapp found that the oxytocic hormone disappeared from the circulation after injection at the same rate as the pressor hormone.

The fate of the chromatophore hormones has been studied even less than that of the oxytocic. After the injection of 40 melanophore units intravenously into human subjects Jores (1936) found that in 15 minutes it had practically disappeared from the blood and only a trace could be detected in the urine. Zondek and Krohn (1932b) could not detect intermedin in normal urine and could not even detect it when added to urine unless the added quantity was very large. While whole urine often exerts a melanophore-dilating action this is not necessarily due to the melanophore hormone (Jores, 1936). Consequently the data of Dietl (1931), Ehrhardt (1932), Drouet and Florentin (1933), and Konsuloff (1934), who have reported its presence in urine on the basis of tests with whole urine (some have even regarded their results as diagnostically significant) are of no value.

XXI. Concluding Remarks

It may not be out of place to terminate this review of the actions of crude extracts and more or less purified fractions of extracts of the posterior lobe of the pituitary gland with some remarks of a general nature.

One feature which characterizes these actions is that all are acute; that is, the effects are apparent soon after the administration. They may be called trigger actions to distinguish them from those of some other hormones which cause morphological changes and whose effects are the consequence of these morphological changes.

Thus they have features in common with the autonomic mediators. Unlike these, however, which are liberated locally and consequently exert local rather than general effects (the discharge of adrenaline from the adrenal glands being an exception), the posterior lobe hormones appear to be liberated into the general circulation and no tissue is spared contact with them. This would seem to indicate that they affect phenomena common to many tissues. In this respect they resemble insulin and thyroxin.

There is a resemblance between the action of the pressor hormone and the autonomic hormones in that it, like them, can produce either contraction or relaxation. Upon the upper colon it causes relaxation, upon the lower part contraction. This sort of phenomenon, which on first consideration appears enigmatical, is not unintelligible if one regards the actions as trigger actions. There is no apparent reason why a substance should not cause relaxation as well as contraction if it merely sets a mechanism in action. Indeed one can imagine that it might be highly inconvenient if the same substance always elicited one effect but never the other. There may be something in the economy of nature, for example, which makes it desirable to cause excitation in one part and inhibition in another by the use of the same mediator. A hypothesis which tries to explain the excitatory and inhibitory actions of acetyl choline, adrenaline and noradrenaline on the basis of these substances being the immediate cause of the effect would seem to be open to more difficulties than a hypothesis which regards them as merely pulling triggers. In the latter case what happens depends only upon the arrangement of the apparatus. A trigger can release a spring or compress it depending upon the machine it activates. One does not need anything like the sympathin hypothesis to explain the contraction or relaxation caused by adrenaline. It is well to remember, however, that scientific explanations rarely if ever get to the last link of any chain of events.

It is a hazardous business to attempt to decide which of the manifold actions discussed above are of physiological significance. It does not follow that because effects may be produced by large doses these effects ever occur in the body as the result of normal posterior lobe activity. But whether they do or not, some of these actions are therapeutically useful; the coronary constricting action of the pressor hormone would seem to be a harmful one. Pituitary extract therefore is a drug as well as a hormone.

In the case of only two of the actions, those of the pressor hormone upon urine secretion and of the melanophore hormone upon color changes in a few lower classes of animals, is there good reason to suppose that any are physiologically operative. It may be that action of the oxytocic hormone is physiologically important. Its effect upon phosphate secretion is produced by such minute quantities that it may have some physiological action concerning mineral metabolism. Other actions have been mentioned which are also produced by very minute quantities of material.

Whether the vascular actions of the pressor hormone are physiological or not is an open question. While doses which produce blood pressure effects are small they are many times larger than those which cause antidiuresis. The possibility that capillary tone is regulated by this hormone is a possibility but nothing more. That the alimentary tract normally is affected by this hormone is also no more than a possibility.

Some of the effects of posterior lobe extracts are doubtless merely those resulting from the severe vascular action of large doses. The diuretic action in dogs and cats belongs in this category and also perhaps the effects upon the oxygen and lactic acid content of blood.

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Steroid Configuration

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I. INTRODUCTION

As a preface, and with a view to intelligibility, in Section II, the present conventions in regard to numbering of the steroid nucleus and side-chain, the lettering of the four rings, and the description of stereoisomerides are set out.

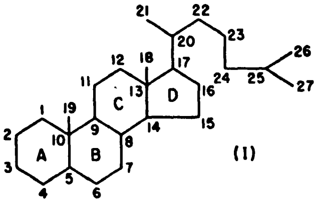
Sections III and IV deal essentially with problems of geometrical isomerism. In Section III, because of the limited number of probabilities the present generally accepted view is given, the evidence for that view is cited, and the special methods affording that evidence given. In Section IV, because of the large range of variations possible, the order of presentation is reversed; general methods are described and illustrated by numerous specific examples.

Section V deals with the less tractable problem of isomerism in the side-chain. This is of the classical tartaric acid type involving "free" rotation; favored constellations, as in ethane, doubtless exist but are unknown, so that the relative configuration, at a given asymmetric center or centers, of a group of related compounds is the maximum progress yet achieved. Such results have been generally obtained by direct correlation or by application of molecular rotation differences.

II. NOMENCLATURE

Conventions for Numbering, Lettering, and Orientation. Absolute Configuration

The carbon skeleton of the hydrocarbons cholestane and coprostane is given in formula (I), which shows the numbering[1] of the system and the alphabetical designation of the four rings.



(I)

The convention adopted for the description and representation of steroids is that originally proposed by Fieser (53), extended by Reichstein and Shoppee (160), and recently modified by Fieser and Fieser (54, 55). Arbitrary trivial indices are indicated by use of "α" and "β." At nuclear centers of asymmetry, position is specified by the number of the carbon atom in question and configuration by the suffixes α or β. If the orientation of the hydrogen atom or a substituent corresponds to that of the angular carbon atom C_{19}, which by convention is regarded as lying above the general plane of the ring system, then the atom or group possesses the β-configuration and this is represented by a full-line bond. Conversely, an atom or group, which is regarded as lying below the general plane of the ring system, is said to possess the α-configuration and this is represented by a broken-line bond. The Greek letters are written without parentheses, and can therefore be used in respect of continental nomenclature e.g., androstandiol-(3α, 11β)-on-17, in which parentheses are normally employed for typographical purposes.

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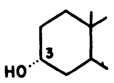
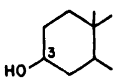
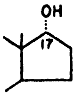
[1] Following recent advances in the chemistry of strophanthidin and "α"-antiarin and the isolation of new glycosides such as cymarol and sarmentoside B, it is most desirable that the carbon atom attached to C_{10} should uniformly be numbered C_{19} as in (I); see Fieser (55) and Reichstein and Shoppee (160), but compare Karrer (101) and Gilman (67).



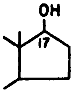
3α-Hydroxy



3β-Hydroxy

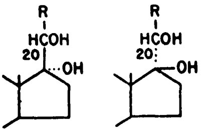


17α-Hydroxy

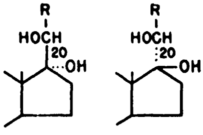


17β-Hydroxy

For asymmetry centers in the C_{17}-side-chain, position is specified by the number of the carbon atom bearing the substituent, but since stereoisomerism is now no longer geometrical in character (fixed position above or below the plane of a ring) but of the classical tartaric acid type ("free" rotation), a definite spatial orientation cannot in general be assigned. In the case of configuration at C_{20} *only*, when this can be related to that at C_{17} and the whole ring system, the suffixes α and β are employed according to the convention proposed by Fieser and Fieser (54, 55) (see p. 293).

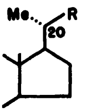


20α-Hydroxy

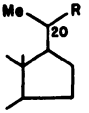


20β-Hydroxy

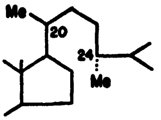
For configuration at C_{20} nonrelative to C_{17}, and at other asymmetry centers in the side chain, the suffixes a and b are employed but *n* and *iso* have also been used. These suffixes serve to distinguish stereoisomerides without any spatial implication, and since suffix assignment is arbitrary, designations applied provisionally in one series imply no correspondence to those in another. Typographically pairs of stereoisomerides may be distinguished by use of full-line and broken-line bonds.



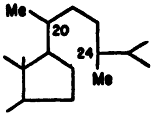
20a or 20-iso



20b or 20-n

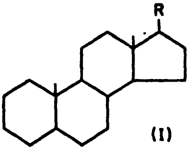


20b: 24a-Dimethyl

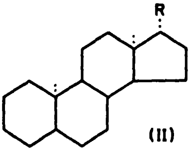


20b: 24b-Dimethyl

Finally, it may be pointed out that the absolute configuration, i.e., with respect to *d*(−)-lactic acid or *d*(+)-malic acid, of any steroid center of asymmetry has not yet been determined (*cf.* however, 20[2]); such a future determination may show the actual arrangement of the steroid nucleus to be the mirror image (II) of that (I) accepted by convention today.



(I)

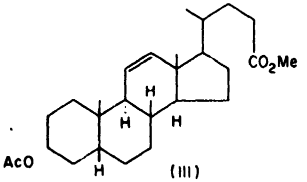


(II)

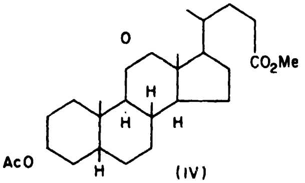
III. The Steroid Nucleus

1. Configuration of the Angular Methyl Group (C_{18}) *at* C_{13}

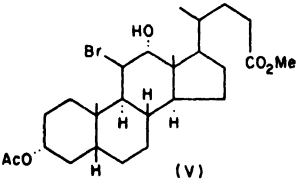
Methyl 3α-acetoxychol-11-enate (III) with perbenzoic acid gives the oxide (IV) (151, 60, 123), which differs from the epimeric oxide obtained indirectly from (III) (155, 137), and which by catalytic hydrogenation in the presence of hydrobromic acid passes through the intermediate bromhydrin (V) to afford derivatives of deoxycholic acid (151, 123). The bromhydrin (V) is also obtained from the oxide (IV) by addition of hydrogen bromide and its structure is known (60, 61, 62, 180) from its conversion by oxidation with chromium trioxide to the 11β-bromo-12-keto-ester (VI), which is identical with that one of the pair of 11-epimerides obtained as the minor product by bromination of methyl 3α-acetoxy-12-ketocholanate (180). The 11β-bromo-12α-hydroxy struc-



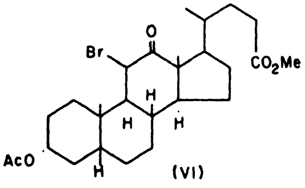
(III)



(IV)



(V)



(VI)

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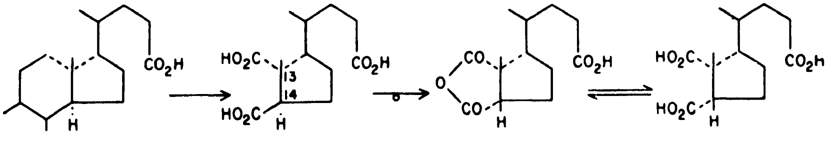
[2] See also A. Lardon and T. Reichstein (*Helv. Chim. Acta* **32**, 1613 [1949]) and S. Bergström, A. Lardon, and T. Reichstein (*ibid.* **32**, 1617 [1949]) who describe the degradation of vitamin D_2 [calciferol] methyl ether to (−)β-methoxyadipic acid.

ture (V) is consistent with the inversion of configuration known to occur on fission of the oxygen-carbon bond in ethylene oxides (6, 223, 227), and with the reactivity of the derived 11β-bromo-compound (VI) as compared with its 11α-bromo-epimeride towards boiling pyridine (60, 2, 180). The oxide (IV), the bromhydrin (V), and deoxycholic acid thus possess the same α-configuration at C_{12} by reference to the center of asymmetry at C_9, the stereochemical relationship of which to the angular methyl group (C_{19}) at C_{10} (9α-H/10β-Me: *trans*) is established (see Section III, 2, b). Since it is known that the hydroxyl at C_{12} in deoxycholic acid is *trans* to the angular methyl group (C_{18}) attached to C_{13} (199), it follows that the latter is *cis* to the angular methyl group (C_{19}) at C_{10}, which by convention is regarded as being β-orientated. The C_{13}-angular methyl group is therefore also β-orientated, and it may be noted that any other arrangement raises difficulty from the X-ray crystallographic standpoint in relation to the packing of steroid molecules into unit cells.

2. The Ring Fusions A/B, B/C, and C/D

a. C/D Ring Unions. There is no evidence from X-ray crystallography (16, 39), but chemical evidence indicates that this ring union is *trans* in the bile acids and in the sterols, and therefore in most steroids, but *cis* in the cardiac glycosides and aglycones and the toad poisons.[3]

In the bile acid series, Wieland and Dane (218) obtained by degradation of deoxycholic acid the *trans*-C_{13}:C_{14}-dicarboxylic acid (VII), which furnishes no anhydride but by heating is converted into the *cis*-C_{13}:C_{14}-dicarboxylic acid anhydride (VIII); this readily affords the *cis*-C_{13}:C_{14}-dicarboxylic acid (IX) on mild hydrolysis, so that the original C/D-ring fusion must have been *trans* (cf. however 138):



Bile acid (VII) (VIII) (IX)

In the sterol series, Dimroth and Jonsson (40) obtained from vitamin D_2 by permanganate oxidation the ketone (X), which by treatment with acid or alkali is converted irreversibly into the isomeride (XI); this change is characteristic of *trans*-"α"-hydrindanones, which are known to undergo prototropic conversion *via* the enol to the *cis*-isomerides (80), and confirms the original *trans*-C/D-ring fusion in ergosterol, with which other sterols have been correlated by conversion to 3β-acetoxy-

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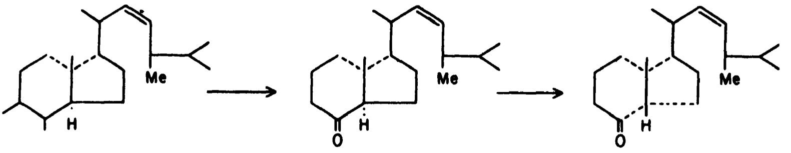
[3] See K. Meyer (*Helv. Chim. Acta*, **32**, 1238, 1593, 1599 (1949)), which appeared after completion of this article.



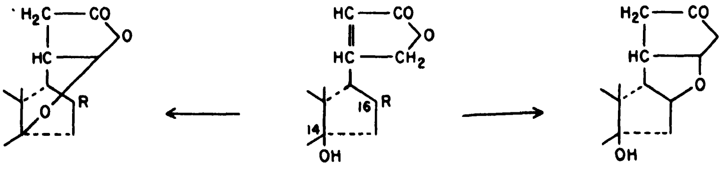
Ergosterol
via vitamin D_2

(X)

(XI)

norallocholanic acid (50, 219). It may be noted that neither the total syntheses of (+)-equilenin (5, 98) nor that of (+)-estrone (3) proves that the C/D ring fusion in these hormones is *trans;* there is however little doubt that this is the case and the chemical evidence has been reviewed (191), while optical rotatory data (*vide infra* p. 280) are also consistent with this view (106).[4]

In the cardiac glycosides and aglycones (XII: R═H) the formation of iso-compounds (XIII: R═H) is possible only if the hydroxyl group at C_{14} and the lactonic side chain at C_{17} are *cis* to one another, or if inversion of configuration occurs at either of these points during the reaction, or, lastly, if the accepted mechanism of the irreversible formation of such iso-compounds is incorrect. The last alternative seems improbable in view of the formation from gitoxigenin (XII: R═OH), which has been proved to possess a C_{16}-hydroxyl group (134), of *two* types of iso-lactone (XIII: R═O) and (XIV) (90). The second alternative is excluded in respect of C_{17} by proof of the β-configuration of the side chain in both aglycones (128, 199, 200, 203) and iso-compounds (91, 94) while in view of the mild conditions under which the change (XII → XIII) occurs, inversion at C_{14} seems unlikely. The established β-configuration of the side-chain requires that the C_{14} hydroxyl group is



(XIII)

(XII)

(XIV)

β-orientated, which is proved correct by the synthesis of methyl 3β-acetoxy-14β-hydroxy-14-isoetiocholanate (173), a degradation product of digitoxigenin (83). It therefore appears certain that the cardiac glycosides and aglycones contain a *cis*-C/D ring fusion. This is also true of the toad poisons, since bufalin has recently been degraded to 3β-acetoxy-14β-hydroxy-14-isoetiocholanic acid, telocinobufagin to 3β-acetoxy-5β:14β-dihydroxy-14-isoetiocholanic acid, and gamabufogenin to 3β:11α-diacetoxy-14β-hydroxy-14-isoetiocholanic acid.[5]

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[4] See W. E. Bachmann and A. S. Dreiding (*J. Am. Chem. Soc.*, **72**, 1323 (1950)) which appeared after completion of this article.

b. B/C Ring Unions. The junction of rings B and C is generally regarded as *trans* because of the requirements of the X-ray crystallographic measurements of Bernal *et al.* (16, 39), which are supported by dipole moments (116), but Bernal states "this determination has not anything like the certainty of that of the parallel case of the *cis*- and *trans*-hexahydrochrysenes. In the completely reduced ring-system there is much greater freedom of movement[6] and models can be constructed with a *cis*-central junction nearly as flat as those where the junction is *trans*. But where one ring is aromatic as in estrone, more rigid conditions hold and here it is certain that the central junction must be *trans*. The relations between the sterol and sex-hormone group justify one in extending this condition to all normal members of the (steroid) series, especially when it is realized that any change of the configuration of the central junction made without corresponding changes at the C/D-junction completely alters the shape of the molecule." Bernal also states that to keep the molecule flat the hydrogen or carbon atom attached to C_{10} must in general be *trans* to the hydrogen atom at C_9; since C_{19} is by convention β-orientated, this further determines that, of the two possible modes of *trans*-B/C linkage (cf. XVIIIAa and XVIIIBa), the mode involving α-configuration for the hydrogen at C_9 is correct.

From a chemical point of view, the stability to $OH^{\ominus}$ of 7:12-diketocholanic acid (216) and 11-ketosteroids indicates a *trans*-B/C ring union; both these compounds may be regarded as an "α"-decalone, the *cis*-form of which undergoes almost complete transformation in the presence of alkali to the *trans*-form (79). The adrenocortical hormones and related synthetic products possess a β-orientated C_{11}-hydroxyl group and are readily dehydrated by mineral acid or by phosphorous oxychloride to afford mainly $\Delta^{9(11)}$-unsaturated compounds (117, 129, 156, 182), while synthetic 11α-hydroxy compounds are resistant to dehydration (61, 62, 185). Ionic elimination reactions, as opposed to thermal elimination reactions (9), require a *trans*-arrangement of the participating atoms or groups, which is consistent with the relationship [9α-H/11β-OH:*trans*] resulting from a *trans*-B/C union. Similarly, 11β-bromo-steroids [9α-H/11β-Br:*trans*], undergo ionic elimination of hydrogen bromide with boiling pyridine whereas the epimeric 11α-bromo-compounds [9α-H/11α-Br: *cis*] are unchanged (2, 60, 180). Conversely, the relationship [8β-H/7α-OH: *trans*] present in cholic acid and cholestane-3β: 7α-diol as the result of *trans*-B/C union is consistent with the ease with which these substances undergo ionic elimination of water to yield mainly Δ^7-unsaturated compounds (21, 57, 69, 217), while cholestane-3β: 7β-diol is resistant to ionic dehydration (57).

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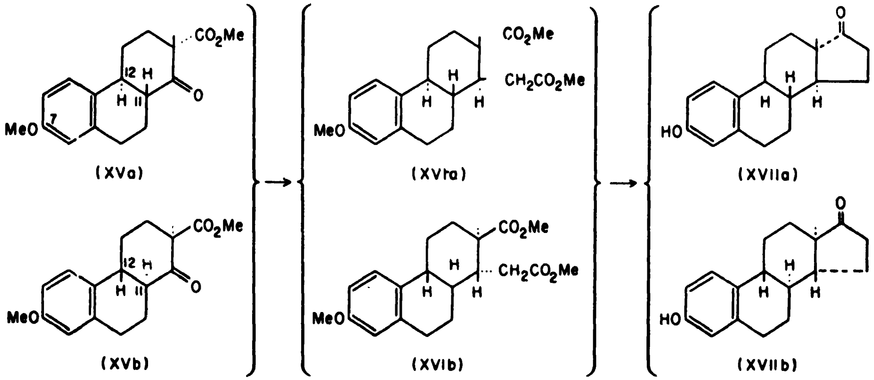
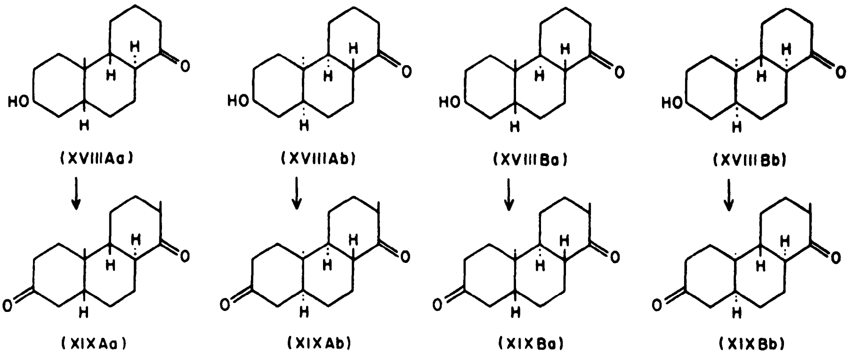
[5] See K. Meyer (*Helv. Chim. Acta* **32**, 1238, 1593, 1599 (1949)).

[6] In boat forms (*vide infra*).

The total synthesis of (+)-estrone (XVIIa) by Anner and Miescher (3) also indicates that in this hormone the B/C-ring fusion is *trans*. In this case the appropriate racemic ketoester (XVa + XVb) was selected for the synthesis, which proceeds *via* the racemic 7-methylmarrianolic acid (XVIa + XVIb), on the basis of evidence that the hydrogen atoms at C_{11} and C_{12} (phenanthrene numbering) have the stereochemical arrangement corresponding to a *trans*-B/C ring fusion at C_8 and C_9 (steroid numbering).



The total synthesis of the diketone (XIXBa) by Cornforth and Robinson (37) provides further evidence for a *trans*-B/C ring union in the sterols and bile acids. The stereoisomerides (XVIIIA) and (XVIIIB), obtained from 1:6-dihydroxynaphthalene, are "α"-decalones and, since they have been subjected to treatment ($OH^{\ominus}$) known to convert *cis*-"α"-decalone into the *trans*-isomeride, represent the two possible modes of *trans*-B/C ring fusion. Resolution affords the enantiomorphs (XVIIIAa and b; XVIIIBa and b) which have been separately transformed into the four diketones (XIXAa and b; XIXBa and b), one of which (XIXBa) has been shown by melting point, mixed melting point, optical rotatory power, and X-ray crystallographic analysis to be identical with the diketone obtained from cholesterol (37, 110) and deoxycholic acid (157).



The suggestion of a *cis*-B/C ring union has been made in two cases only: (i) sarmentogenin (209) has now been shown to be a 3β:11α:14β-trihydroxy compound with a *trans*-B/C linkage by degradation to methyl 3β:11α-diacetoxyetiocholanate (103); (ii) the urane compounds of Marker *et al.* (123), which Klyne (107) has recently proved to be derivatives of 17-methyl-D-homoandrostane and so to contain a B/C: *trans* linkage.

c. A/B Ring Unions. The stereochemical relationship of the four hydrocarbons cholestane, allocholane, allopregnane, and androstane (A/B:*trans*) to their isomerides coprostane, cholane, pregnane, and 5-isoandrostane (A/B:*cis*) is firmly established. The original proof involved lithobilianic-(3||4), isolithobilianic-(2||3), and allolithobilianic-(3||4) acids and was adduced by Windaus (225); the proof is set out in Fieser and Fieser (55) and need not be given here.

The identity of the synthetic diketone (XIXBa), which has been proved to contain a *cis*-A/B-ring fusion (37), with the compound obtained by degradation of deoxycholic acid constitutes further evidence that the bile acids involve *cis*-A/B ring union.

3. Geometry of Rings A, B, C and D

The *cyclo*hexane ring can exist theoretically in two strainless forms—the chair-form, which is rigid, and the boat-form, which is highly mobile; the chair-form can undergo conversion into the boat-form by passage over a small energy barrier calculated to be < 10 kcal. (116, 186). The steroid nucleus contains three fused *cyclo*hexane rings, but owing to interlocking such chair-boat transformation appears possible in the intact nucleus only at C_{12} in ring C (*vide infra*, p. 264), and at C_3 in ring A (*vide infra*, p. 264).

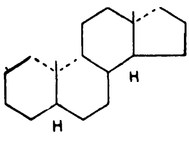
a. Rings C and D. Although a five-membered carbocyclic ring is generally regarded as planar, it has been shown that the mutual repulsions of the hydrogen atoms in *cyclo*pentane cause slight puckering (4). If rings C and D are *trans*-united, because C_{18} is β-orientated, then ring D is puckered with C_{15} in front, C_{17} behind, and C_{16} approximately in the plane of C_9, C_{11}, C_{13} and C_{14} (see XX) (*cf.* also 39). A further consequence is that ring C *must* be a chair-form (which is rigid) with C_{12} lying behind the plane of C_9, C_{11}, C_{13}, and C_{14}, and with C_8 lying in front of that plane owing to locking by ring B (*cf.* XX).

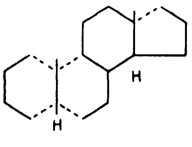
When rings C and D are *cis*-fused, as in the cardiac glycosides and aglycones, C_{18} is β-orientated and normal to the plane of C_9, C_{11}, C_{13}, and C_{14}, but *only* if ring C is a rigid chair-form; ring D is puckered and lies behind and at 60° to the plane C_9, C_{11}, C_{13} and C_{14}.

With *cis*-fusion of rings C and D, ring C can also be a boat-form with both C_{12} and C_8 lying in front of the plane of C_9, C_{11}, C_{13} and C_{14}; C_{18} is still β-orientated but is no longer normal to the plane of C_9, C_{11}, C_{13} and C_{14}, while ring D is now planar and normal to the plane of C_9, C_{11}, C_{13} and C_{14}. These geometrical possibilities may be reflected in reactivity at C_{12} in cardiac aglycones such as digoxigenin, and in the toad poisons arenobufogenin (221), cinobufagin (111), and cinobufotalin (112) if these substances prove to possess hydroxy or acetoxyl groups at C_{12}.

b. Ring B. Since C_{19} is by convention β-orientated, it follows that C_9 must lie behind the plane of C_5, C_7, C_8 and C_{10} and it is locked in this position by *trans*-union of rings B and C.

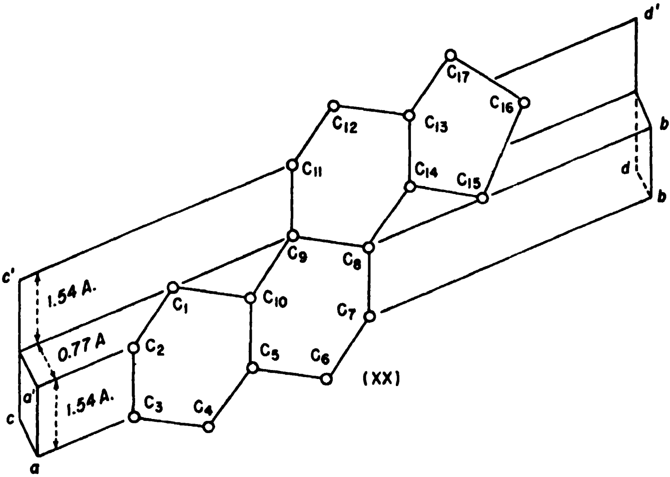
c. Rings A and B. Since C_{19} is by convention β-orientated, there are two immediate possibilities originally suggested by Ruzicka, Furter and Thomann (164, cf. 165) for the configuration of rings A and B, namely (XX) for cholestane, allocholane, allopregnane, and androstane, and (XXI) for coprostane, cholane, pregnane, and 5-isoandrostane.





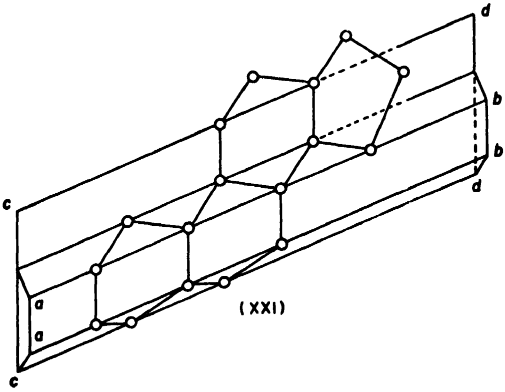
Because the chair-form of *cyclo*hexane is rigid, it might be thought that (XX) is a rigid structure; ring A can, however, undergo conversion into a boat-form, by relative motion of C_2, C_3 and C_4 whereby C_3 and C_{10} become the ends of the boat, without disturbance of the remainder of the tetracyclic structure (186).

cis-Union of rings A and B suggests that both these rings are boat-forms as in (XXI), which at first sight preserves the lathlike shape, disclosed by x-ray crystallographic studies (16), of molecules based on (XX); the boat form of *cyclo*hexane is, however, a mobile structure, and rings A and B in (XXI) are capable of crumpling to an astonishing degree. The 3α:9α-epoxy-compounds of Kendall *et al.* (130, 212) are readily derived using normal bond lengths for the C_3—O—C_9 linkage if rings A and B of the parent cholanes are both boat-forms (192); this is



(XX)

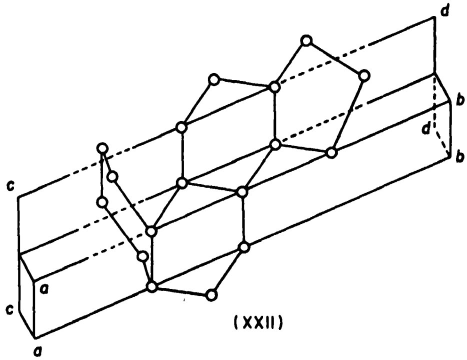
not so in the case of the L-shaped modification (XXII) based on a structure proposed for *cis*-decalin by Bastiansen and Hassell (12), in which despite *cis*-union both rings A and B are chair-forms, but again becomes the case when ring A of (XXII) becomes, by relative motion of



(XXI)

C_2, C_3 and C_4, a boat-form with ends at C_3 and C_{10}, and ring B remains a chair-form the L-shape being preserved (8, 165, 188).

It may be noted that on energetic grounds, the double chair structure (XXII) would be expected to be more stable than the double boat structure (XXI)(188) by about 5 kcal. (8), although existing x-ray crystallographic evidence for coprostane (16) and cholane compounds (16, 66) suggests that these are flat and not L-shaped.



(XXII)

The energies which might be expected to be involved in geometrical transformations of the types discussed above are probably of the order of 5–10 kcal., which is small compared with the activation energies of most chemical reactions, and they therefore appear to be derivable from thermal bombardment at ordinary temperatures. Stereochemistry deals with structures which retain individuality under such conditions (222); from the stereochemical point of view, it is useless to attempt to distinguish between geometrical arrangements which are separated by energy barriers so low that they will normally be traversed with great frequency in the liquid state or in solution. Very many substitution products of androstane, 5-isoandrostane, and their homologues are known, but there is no established case of the existence of more than the two epimeric nuclear substitution products predicted on the basis of formulae (XX) or (XXI).

The various possible geometrical modifications of the nuclei (XX) and (XXI) thus appear to make no contribution to steroid stereochemistry. Such geometrical modifications cannot affect the character (*cis*- or *trans*-) of a ring-fusion already present in a molecule, but may affect reactivity (e.g., equilibria and reaction rates). Thus in a $5\alpha:6\beta$-diol of the androstane, allopregnane, allocholane or cholestane series (ring B = chair-form), the hydroxyl groups will be too distant to permit complex formation, and Fieser (58) has found that such a glycol is resistant to periodate oxidation at 20°; in a $5\beta:6\alpha$-diol of the 5-isoandrostane, pregnane, cholane, or coprostane series (ring B = boat-form) with constellation as in (XXI), the hydroxyl groups are orientated in opposite directions, but because the boat-form is mobile, although mobility is restricted by union with ring C, a constellation is possible and achieved without strain in which the hydroxyl groups appear not too distant for complex-formation, so that here the 5β:6α-diol and the 5β:6β-diol should be approximately equally capable of periodate oxidation.[7]

IV. Configuration of Nuclear Substituents

General Methods for the Determination of Configuration

General methods which have been used to determine the configuration of nuclear substituents and examples of their use, are as follows:

a. Formation of a Cyclic Compound (1) between Substituents at Non-adjacent Carbon Atoms. The limitation to nonadjacent carbon atoms (188) arises from the mobility of the boat-form of a *cyclo*hexane ring; this expresses itself in the ability of both *cis-* and *trans-cyclo*hexane-1:2-dicarboxylic acid to give anhydrides. This limitation obviously does not apply to substituents in ring D.

A substituent at a bridge head, e.g., C_5 or C_{14}, is often chosen to form one end of a cyclic structure arising from interaction with another nuclear substituent because its orientation follows from the character, *cis-* or *trans-*, of the ring union concerned; this is convenient but not essential.

It should be noted that it is possible for two substituents to be β-orientated and suitably distant for ring-formation, but to fail to interact owing to the intervention of the C_{13}-angular methyl group. Thus 3α:12β-dihydroxyetiocholanic acid [12-epietiodeoxycholic acid] (190, 199) and 3β-acetoxy-14β-hydroxy-14-isoetioallocholanic acid (172) fail to give 17 → 12- and 17 → 14-lactones respectively. Conversely, a side chain, regarded as a 17-substituent, must be short to permit conclusions to be drawn in regard to the orientation of another nuclear substituent interacting with it to afford a cylic compound. Thus, although the 12-hydroxyl group is α-orientated and the side chain β-orientated, deoxycholic acid (214, 215) (cf. 2 and 27) and nordeoxycholic acid (141) furnish 24 → 12- and 23 → 12-lactones respectively, but this becomes impossible in bisnordeoxycholic acid (108) and etiodeoxycholic acid (199).

LACTONE FORMATION

C_3:C_5. The hydroxy-dibasic acid (XXIII) readily yields a 5 → 3-lactonic acid (XXIV) (118, 192) which has bonds of normal length

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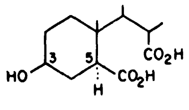
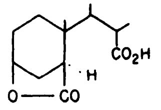
[7] Since this article was written, D. H. R. Barton (*Experientia,* 1950, **6,** 316) has applied the concept of "polar" and "equatorial" bonds in *cyclo*hexane rings (K. S. Pitzer and C. W. Beckett, *J. Am. Chem. Soc.,* 1947, **69,** 977, 2488; cf. O. Hassel and H. Viervoll, *Acta Chem. Scand.,* 1947, **1,** 149, 929) to the interpretation of steroid equilibria and reaction rates, and to the deduction of configuration (see also D. H. R. Barton and E. Miller, *J. Amer. Chem. Soc.,* 1950, **72,** 1066).

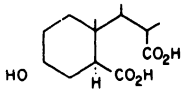
only if the *cyclo*hexane ring (formerly ring A of the nucleus) is a boat-form (192); the 3-epimeride (XXV), affords the unsaturated anhydride (XXVI). The formation of a γ-lactone from (XXIII) *only* indicates that the C_3-hydroxyl and the C_5-carboxyl lie on the same side of the ring, and since the latter is β-orientated (cholestane series) the former must also possess the β-configuration.



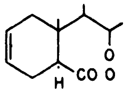
(XXIII)



(XXIV)

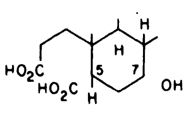


(XXV)

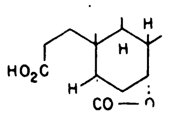


(XXVI)

C_5:C_7. The hydroxy-dibasic acid (XXVII), derived from chenodeoxycholic acid, is readily converted into the 5 → 7-lactonic acid (XXVIII) (24, 224), which has bonds of normal length only if the *cyclo*hexane ring (formerly ring B of the nucleus) is a chair-form (192). Both the C_7-hydroxyl and the C_5-carboxyl lie on the same side of the ring, and since the latter is α-orientated (cholane series) the former must likewise possess the α-configuration.

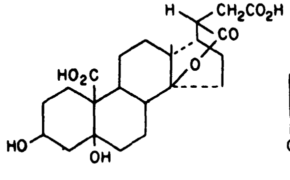


(XXVII)

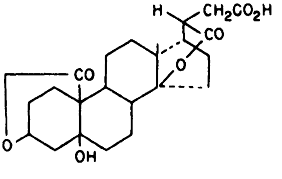


(XXVIII)

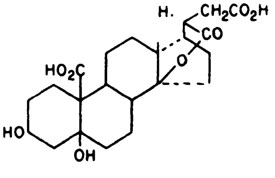
C_3:C_{19}. "β"-Isostrophanthic acid, a 21 → 14-lactone (XXIX), readily affords a 19 → 3-dilactone (XXX) which suggests that, as in periplogenin (200, 201), the 3-hydroxyl group is β-orientated (45). The isomeric "α"-isostrophanthic acid (XXXI) does not form a 19 → 3-dilactone (89); the reason for this is obscure, because (XXIX) is obtained by treatment of the C_{19}-aldehydic precursor of (XXXI) with sodium hydroxide and subsequent oxidation with sodium hypobromite, and it seems probable that the "α" and "β" compounds are C_{20}-stereoisomerides (55) although other possibilities e.g., inversion at C_3 or C_5 have been suggested (45, 208). The 19 → 3-lactones derived from



(XXIX)



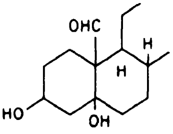
(XXX)



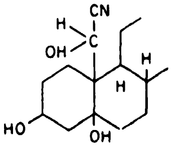
(XXXI)

dianhydrodihydrostrophanthidin are best discussed in connection with the related C_3:C_{19}-oxides (see page 271).

C_5:C_{19a}.[8] Dihydrostrophanthidin (XXXII) affords a mixture of C_{19}-epimeric cyanhydrins (XXXIII) hydrolyzed by acetic acid to two homoacids, which lactonize to give respectively two 19a → 5-homolactones represented by (XXXIV), which are C_{19}-epimerides since by oxidation both furnish the same 3:19-diketohomolactone (XXXV) (96). Since C_{19} is by convention β-orientated, the 5-hydroxyl must also be β-orientated (cf. 144). $\Delta^{14(15)}$-Unsaturated analogues of the 14β-hydroxy compounds (XXXIV) and (XXXV) have been described (96).



(XXXII)



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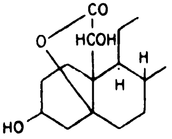

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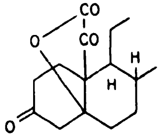
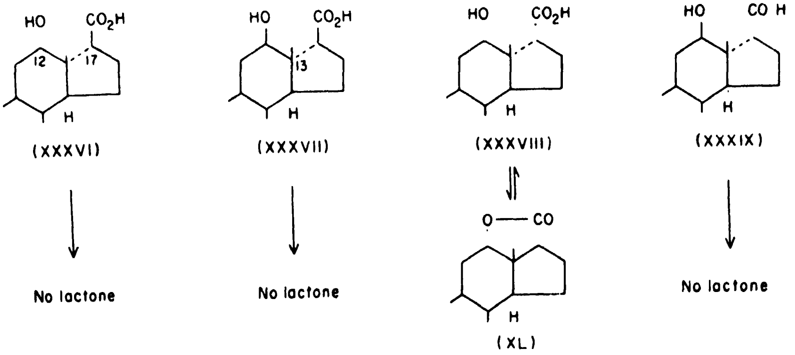


(XXXV)

C_{12}:C_{17}. Sorkin and Reichstein (199) have examined the capacity for γ-lactone formation of the four etiodeoxycholic acids differing in configuration at C_{12} and C_{17} (XXXVI, XXXVII, XXXVIII, XXXIX). Only 17-isoetiodeoxycholic acid (XXXVIII) yields a 17 → 12-lactone (XL) and it does so with extreme ease; the C_{12}-hydroxyl and the C_{17}-carboxyl group thus lie on the same side of the general plane of the C/D-ring system, so that if the former is α-orientated, the latter must possess the α-configuration in (XXXVIII) and the β-configuration in etiodeoxycholic acid (XXXVI) and in the natural steroids. 12-Isoetiodeoxycholic acid (XXXVII) might have been expected to lactonize under sufficiently drastic conditions, but this is not the case (199);



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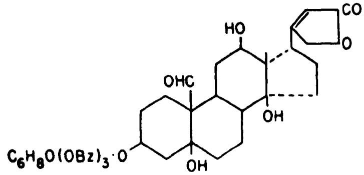
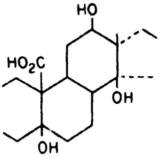
[8] The extra carbon atom in the C_{19}-side chain is referred to as C_{19a}.

apparently the β-orientated C_{18}-methyl group constitutes an insurmountable obstacle (190).

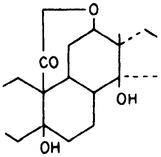
C_{12}:*C_{19}*. The conversion of "α"-antiarin tribenzoate (XLI) by oxidation with chromium trioxide into a lactone suggests that ring closure occurs between the carboxyl group involving C_{19} and a β-orientated hydroxyl group at C_{11} or C_{12}, of which the latter alternative is preferred (43) as in (XLII) → (XLIII).



(XLI)

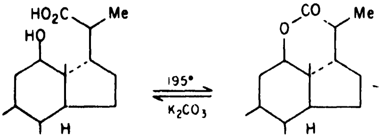


(XLII)

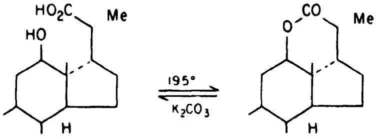


(XLIII)

C_{12}:*C_{21}*. 12-Epi-20-*n*-bisnordeoxycholic (XLIV) and its 20-isoepimeride (XLVI) afford 21 → 12 lactones (XLV) and (XLVII) respectively, which by hydrolysis regenerate their parent acids; these results enabled Sorkin and Reichstein (197) to show that the 12-hydroxyl group and the side chain in (XLIV), (XLVI) must be on the same side [now known to be β] of the general plane of the ring system.



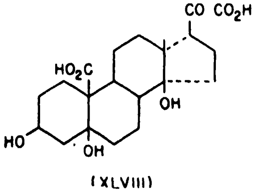
(XLIV) (XLV)



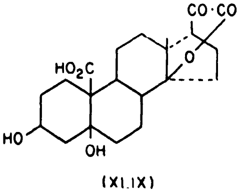
(XLVI) (XLVII)

C_{14}:*C_{19}*. The supposed 14 → 19-lactones derived from strophanthidinic acid, and their analogues, are considered in connection with the related C_8:C_{19}-oxides (*vide infra*, page 272).

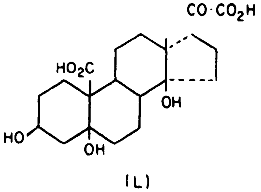
C_{14}:*C_{21}*. The ketonic dibasic acid (XLVIII) obtained by oxidation of strophanthidinic acid (33, 84) passes at once into the 21 → 14-lactone (XLIX); treatment of (XLVIII) with $OH^{\ominus}$ gives the 17-iso-keto acid (L), which does not afford a lactone (84). The C_{20}-carboxyl and C_{14}-



(XLVIII)



(XLIX)

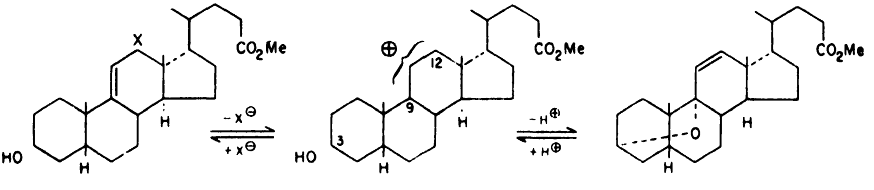


(L)

hydroxyl must lie on the same side of the general plane of the C/D-ring system in (XLVIII); since the former is part of a β-orientated C_{17}-side chain, the latter must possess the β-configuration. For another example, see Helfenberger and Reichstein (75).

OXIDE FORMATION

C_3:C_9. In anhydrous nonpolar solvents methyl 3α-hydroxy-12-halogenochol-9(11)-enate (LI: X=Cl or Br) is stable, but the halogen is rapidly and completely removed by treatment with water at 20° to give, *via* the mesomeric cation (LII), the 3:9-oxide (LIII) (130, 133, 212);

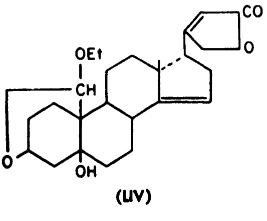


(LI) (LII) (LIII)

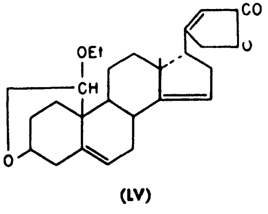
the acetate of (LI) is stable under these conditions. Since ether formation does not involve inversion of configuration (82, 105) and since the formation of an oxide bridge between C_3 and C_9 in the presence of the β-orientated methyl group at C_{10} can only occur if the oxide is a 3α:9α-oxide, the hydroxyl at C_3 in (LI) and hence in the bile acids generally must be α-orientated. The formation of 3α:9α-oxides with normal bond lengths for the C—O—C linkage affords information about the geometry of rings A and B in the cholane nucleus (8, 192).

The stability of the 3α:9α-oxide bridge is considerable (212) and survives Wieland-Barbier degradation of the side chain (133), but treatment with hydrochloric or hydrobromic acid causes fission, e.g., (LIII) → (LI).

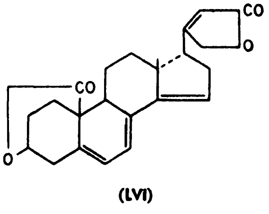
C_3:C_{19}. Strophanthidin with cold alcoholic hydrogen chloride affords the cyclic acetal of anhydrostrophanthidin (LIV), while hot acid furnishes the cyclic acetal of dianhydrostrophanthidin (LV) (85), which by oxidation with chromium trioxide passes into the lactone (LVI) accompanied by some of the corresponding 3-hydroxy-19-carboxylic acid (87, 97). Oxide ring formation between C_{19}, which is, by



(LIV)

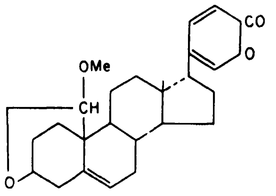


(LV)

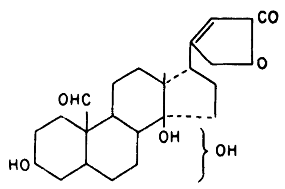


(LVI)

convention, β-orientated, and the 3-hydroxyl group necessitates that the latter has the β-configuration. Various compounds derived from strophanthidin with modification of the nuclear structure of the lactonic side chain also afford 3:19-oxido compounds. Similarly, hellebrin by hydrolysis with methanolic hydrogen chloride yields a methoxy compound formulated as the 3:19-oxide (LVII) (102, 179); adonotoxigenin, formulated as (LVIII) (104), may also be expected to afford 3:19-oxido derivatives if the hydroxyl at C_3 is β-orientated.

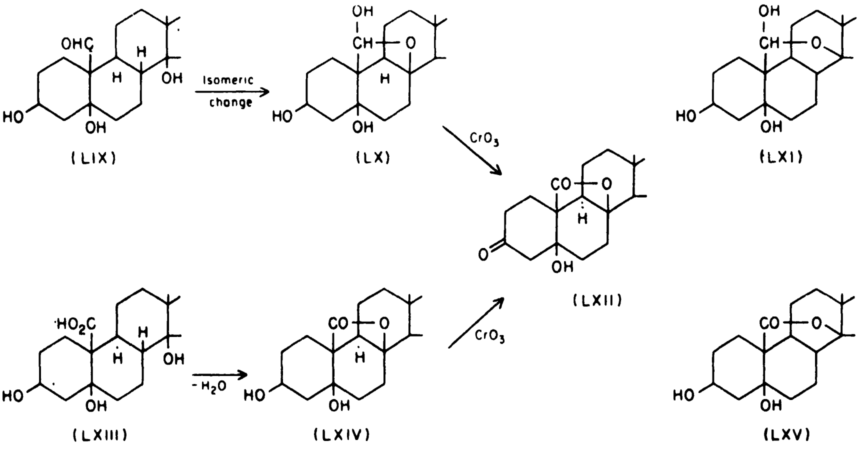


(LVII)



(LVIII)

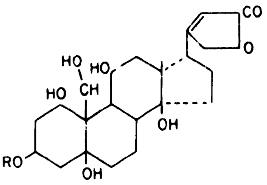
$C_8:C_{19}$. Strophanthidin (LIX) by treatment with hydrochloric acid is isomerised to ψ-strophanthidin (LX), originally formulated as the 14:19-oxide (LXI), which is converted by chromium trioxide into the 3-ketodilactone (LXII) (88); strophanthidinic acid (LXIII) with concentrated hydrochloric acid loses water to give the dilactone (LXIV) (86, 87), originally formulated as (LXV); the 8 → 19-lactone ring is very stable and oxidation gives (LXII). Ring-formation as in (LXI) or (LXV) would normally be regarded as evidence of interaction between two β-orientated groups, but such a 14:19-oxide or 19 → 14-lactone would require bonds of abnormal length in the C—O—C linkage because of the rigidity of the trans-B/C union (cf. 94). Formulae (LX) and



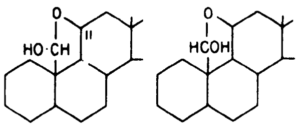
(LIX) (LX) (LXI) (LXII) (LXIII) (LXIV) (LXV)

(LXIV) involving an 8:19-linkage (cf. 55) with bonds of normal length are therefore more probable, and, while the mechanisms of their respective formation are obscure, appear to preclude inversion at C_8 but not at C_{14}. Compare also the anhydro-19 → 8, 23 → 21-dilactone from oxidomonoanhydrodihydrostrophanthidinic acid (95).

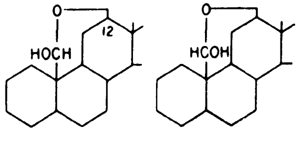
$C_{11}:C_{19}$ *or* $C_{12}:C_{19}$. Ouabain (LXVI: R=$C_6H_{11}O_4$—) by catalytic dehydrogenation with platinum and oxygen gives two isomeric dehydroouabains, regarded by Mannich and Siewert (120) as epimeric 11:19-oxides. If ouabain possesses an 11-hydroxyl group, then this must be β-orientated to furnish the epimerides represented by (LXVII) and should be nonacylatable and sensitive to acids; a β-orientated 12-hydroxyl group giving rise to epimeric 12:19-oxides (LXVIII) would appear to be more consistent with present knowledge (135, 153). If "α"-antiarin (XLI) possesses a 12β-hydroxyl group (43) it might be expected to afford epimeric oxides analogous to (LXVIII), whereas with possession of a 12α-hydroxyl group this would appear to be difficult.



(LXVI)

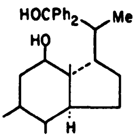


(LXVII)

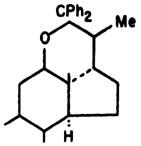


(LXVIII)

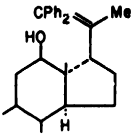
$C_{12}:C_{21}$. Dehydration of the diphenylcarbinol (LXIX) derived from methyl 12-epi-20-*n*-bisnordeoxycholate gives the 12β:21-oxide (LXX) together with the olefin (LXXI) (198); the C_{12}-hydroxyl group and the side chain therefore must lie on the same side (now known to be β) of



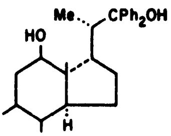
(LXIX)



(LXX)



(LXXI)

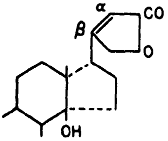


(LXXII)

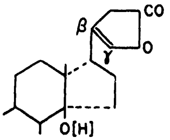
the general plane of the nucleus. It may be noted that the 20-isocarbinol (LXXII) derived from methyl 12-epi-20-isobisnordeoxycholate by dehydration furnishes the same olefine (LXXI) but fails despite "free rotation" to yield the oxide (LXX).

The 12:21-oxides [on Stoll's formulation (205, 206)] derived from scilliroside would appear to require the participation of a 12β-hydroxyl group.

C_{14}:C_{21}. The 14β:21-oxides, known as isocompounds, formed generally by the strophanthus-digitalis glycosides and aglycones, have already been mentioned (p. 260). Their irreversible formation in the presence of $OH^{\ominus}$ or $OMe^{\ominus}$ is due to triad prototropy [αβ-βγ-change] (LXXIII → LXXIV) followed by an intramolecular addition reaction (LXXIV → LXXV) (183); this can only occur when both the C_{14}-hydroxyl group and the C_{17}-side chain are β-orientated. The so-called "alloglycosides" and "alloaglycones" (LXXVI), in which the lactonic C_{17}-side chain is α-orientated (200) do not afford iso compounds.



(LXXIII)



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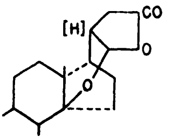

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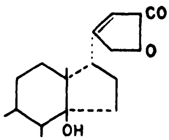
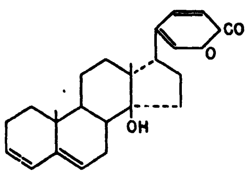


(LXXVI)

The squill aglycone scillaridin A (LXXVII) (204) by treatment with $OMe^{\ominus}$ undergoes cleavage of the δ-lactone ring to give the methyl ester of scillaridinic acid (LXXVIII) which readily passes into the stable 14β:21-oxide ester (LXXIX).



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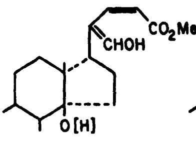

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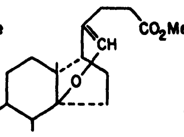

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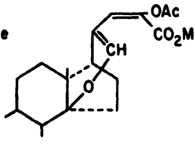


(LXXX)

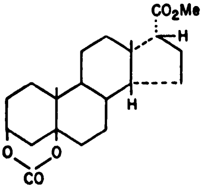
An analogous 14β:21-oxide-ester formulated by Stoll as (LXXX) is obtained from ketoscilliroside (205, 206), and similar 14β:21-oxides are furnished by the toad poisons bufotalone (220), gamabufogenin (113), marinobufagin (207), and cinobufagin (114). These observations indicate that the 14-hydroxyl group and the δ-lactonic side chain are both β-orientated.

C_{16}:C_{21}. Gitoxigenin possesses, in addition to the 14β-hydroxyl group, a secondary hydroxyl group at C_{16} (XII: R=OH) (90, 93); it yields not only 14β:21-oxides (XIII: R= :O) but also 16:21-oxides (XIV). The formation of the latter by a reaction sequence analogous to (LXXIII → LXXIV → LXXV) indicates that the 16-hydroxyl group must be β-orientated. Calotropin affords two series of isocompounds which are regarded as 14β:21 and 16β:21-oxides (76). An alternative formulation of the 12:21-oxides derived from scilliroside, suggested by Fieser and Fieser (55), regards them as 16β:21-oxides.

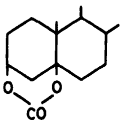
C_{16}:*C_{22}*. The C_{17}-side chain in the saponins and sapogenins is β-orientated, as is shown, for example, by the conversion of diosgenin into cholesterol (127). It follows that the C_{16}—O linkage in the sapogenins and their derivatives possessing a 16:22-oxide structure is β-orientated, and that the triols obtained by oxidation of a sapogenin with Caro's acid (126), and by degradation of the related ψ-sapogenin are 3:16β:20-triols (77).

CARBONATE FORMATION

The available examples all relate to 3:5-carbonates obtained from diols by treatment with phosgene; the first to be described was the 3β:5β-carbonate (LXXXI) obtained by Speiser and Reichstein (200) by degradation of alloperiplogenin. Other examples are the 3β:5β-carbonate from coprostane-3β:5β-diol (LXXXII) (147) and the 3α:5α-carbonate from cholestane-3α:5α-diol (LXXXIII) (145). Clearly, production of such cyclic carbonates proves the *cis*-relationship of the participating hydroxyl groups, and the cholestane-3β:5α-diol (LXXXIV) fails to give a cyclic ester (145).



(LXXXI)



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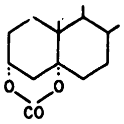

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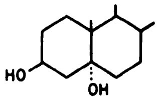
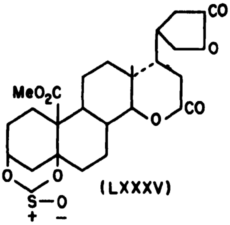


(LXXXIV)

SULFITE FORMATION

The available examples involve 3:5-sulfites obtained from derivatives of strophanthidin by use of thionyl chloride: the ester-sulfite described by Jacobs and Elderfield (92) and the dilactonic sulfite (95) are to be formulated as 3:5-sulfites (LXXXV) and (LXXXVI). Their formation and that of the anhydrostrophanthidin sulfite (LXXXVII) (144) clearly indicate the *cis*-relationship of the hydroxyl groups in the parent 3β:5β-diols.



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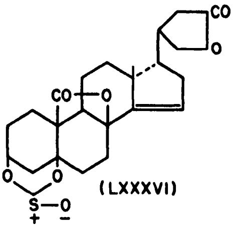

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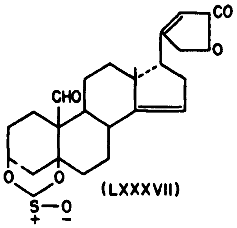
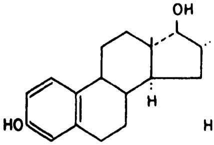


(LXXXVII)

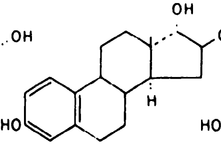
ACETONIDE FORMATION

Ouabagenin (LXVI: R=H) contains numerous nuclear hydroxyl groups, is stable to lead tetracetate, and forms an acetonide (120); an $\alpha\gamma$-diol structure has been suggested, in which the hydroxyl groups must be *cis* to one another.

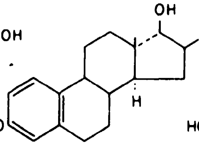
Estriol does not furnish an acetonide (32) but 16-epiestriol (isoestriol A) does so (81); in ring D the limitation of ring-formation to groups on nonadjacent carbon atoms does not apply, so that estriol must possess one of the two *trans*-16:17-diol configurations (LXXXVIII) (LXXXIX), and 16-epiestriol one of the two *cis*-16:17-diol configurations (XC), (XCI). Since estriol is now regarded as (LXXXVIII),[9] 16-epiestriol is (XC), whilst a third isomeride obtained by hydroxylation of a 16:17-double bond with osmium tetroxide must possess the structure (XCI) although there is no information as to its capacity for acetonide formation (168).



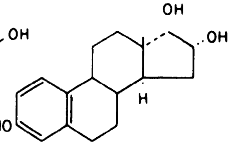
(LXXXVIII)



(LXXXIX)



(XC)



(XCI)

b. Fission of a Cyclic Compound

OSMIC ACID COMPLEXES

The addition of osmium tetroxide to a nuclear double bond gives a cyclic ester which by hydrolysis affords a *cis*-diol which may however be either an $\alpha:\alpha$-diol or a $\beta:\beta$-diol. A multiplicity of examples are available, and it seems probable that use of potassium permanganate also leads to *cis*-diols; thus cholesterol with both reagents gives cholestane-$3\beta:5\alpha:6\alpha$-triol.

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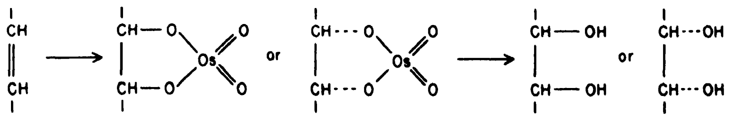
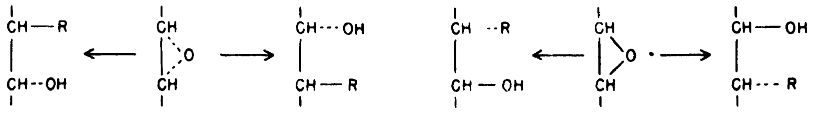
[9] In the original papers (81), the old and incorrect formula for estriol is used, so that the configuration assigned needs reversing.



ETHYLENE OXIDES

Fission of an ethylene oxide involves inversion of configuration at the carbon atom at which rupture of the C—O bond occurs;[10] two isomeric *trans*-forms may result from an oxide although often one form preponderates or is produced almost exclusively:



The cleavage of the α- and β-oxides of cholesterol by hydrogenation, hydrolysis, acetolysis, or with hydrochloric acid (R=H, OH, OAc, Cl) to products of known structure has enabled their configurations to be established as 5α:6α-oxidocholestan-3β-ol and 5β:6β-oxidocoprostan-3β-ol respectively (143).

The reductive fission of various 4:5- and 5:6-oxides has been examined (74, 164, 167), and more recently, re-examined by Plattner *et al.* (146); under catalytic conditions, the reductions generally afford mixtures of various products, so that caution is required in the stereochemical interpretation of the results. It has, however, been found by Plattner *et al.* that reduction by lithium aluminium hydride gives homogeneous products, e.g., 4β:5β-oxidocoprostane-3α-ol and 4β:5β-oxidocoprostane-3β-ol give respectively 3α:5β- and 3β:5β-dihydroxycoprostane (147, 149, 150), which are the first 5β-hydroxysteroids to be synthesized.

The utility of oxides in determining configuration is well illustrated by the epimeric 11:12-oxides from various chol-11-enic acids. The oxides obtained by the action of perbenzoic acid (27, 60, 132, 151) undergo hydrogenation to 12α-hydroxy compounds and are thus 11α:12α-oxides (17, 137); the epimerides obtained by addition of hypobromous acid and subsequent elimination of hydrogen bromide by filtration through aluminium oxide (17, 137) must be 11β:12β-oxides by exclusion and the β-configuration of the 11-hydroxyl group of the adreno-cortical substances follows from this.

The acetolysis of 11α:12α-oxides to 11β-acetoxy-12α-hydroxy-compounds (60, 61) may be noted; reference may also be made to 14β:15β-oxides, which are hydrogenated to 14β-hydroxy-compounds (169, 170, 173), while the 14α:15α-epimerides are resistant to hydrogenation (172), and to the synthesis of the 17α-hydroxy cortical substances J and O by hydrogenation with lithium aluminium hydride of a 16α:17α-oxide (100, 148). For 17:20- and 20:21-oxides, see Section V, 2. Fission of the 16:22-oxide ring of the sapogenins, e.g., tigogenin, has been used to obtain genuine 16β-hydroxy-steroids (77).

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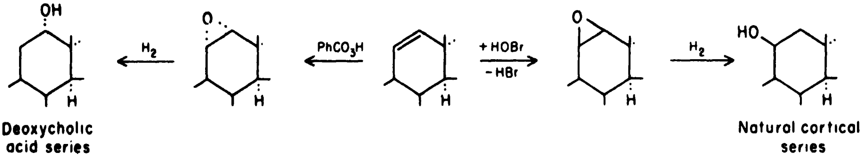
[10] Inversion also occurs on oxide formation, so that a *trans*-orientation of the participating groups is indicated.



c. Molecular Rotation Differences. Analysis of optical rotatory data (59) has frequently been applied to the elucidation of gross molecular structure, e.g., location of double bonds (18, 34). Barton has recently perfected a method for the analysis of molecular rotation differences (7, 10); an excellent general account of this has been given by Fieser and Fieser (55), who have estimated the individual molecular rotation contributions of the eight asymmetric centers at C_5, C_8, C_9, C_{10}, C_{13}, C_{14}, C_{17} and C_{20}, which form a symmetrical and well neutralized pattern: $- - - + - + + +$, with values of $-10°$, $+90°$, $-5°$, $-5°$, $+90°$, $-110°$, $+35$[11], and $+5°$.

Attention here will be confined to application of molecular rotation differences to the determination of nuclear configuration. The following Tables I, II, and III are reproduced in part with the kind permission of Professor and Mrs. Fieser. Table I shows the differences in molecular rotation due to epimerization at a single asymmetric center involving a bridge head; the experimental error in the determination of specific rotation is 1–3° and $[M]_D$ is uncertain to the extent of 5–10 units, so that the uncertainty in $[M^\beta - M^\alpha]_D$ is of the order of ± 10 units.

Provided that the magnitude of the difference $[M^\beta - M^\alpha]_D$ at a single center is sufficient to be diagnostic and that due regard is paid to the possibility of vicinal action, rotational data often permit inferences to be drawn in regard to configuration.

As an example, the rotational evidence indicating the correspondence in configuration at C_{13} and C_{14} of (+)-estrone and (+)-equilenin may be cited (106, 189). From Table I (Nos. 7, 8) it is clear that inversion

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[11] Later results for C_{17} (36) show this value, previously estimated by difference as $+35°$, to be $\sim +75°$.

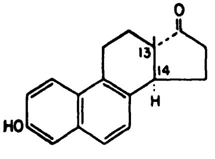
TABLE I

Molecular Rotation Differences Due to Inversion at a Single Center C_x Involving a Bridge Head: $x = 5, 8, 9, 10, 13, 14$, and also 17

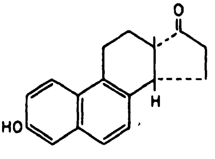
	Epimerides	C_x	$[M]_D$	$[M^\beta - M^\alpha]_D$
1	Coprostanol	5β	+109 C	+16
	Cholestanol	5α	+93 C	
2	epiCoprostanol	5β	+123 C	+14
	epiCholestanol	5α	+109 C	
3	Estrone	8β	+445 C	+191
	8-isoEstrone	8α	+254 C	
4	Estradiol-3:17β	8β	+220 E	+171
	8-isoEstradiol-3:17β	8α	+49 D	
5	isoPyrocalciferol	9β	+1310 C	+1825
	Ergosterol	9α	−515 C	
6	Ergosterol	10β	−515 C	−1275
	lumiSterol	10α	+760 A	
7	Estrone	13β	+445 C	+561
	lumiEstrone	13α	−116 D	
8	Androsterone	13β	+275 E	+565
	lumiAndrosterone	13α	−290 E	
9	Estradiol-3:17β	13β	+220 E	+176
	lumiEstradiol-3:17β 3-Me ether	13α	+44 C	
10	(+)14-isoEquilenin	14β	+391 D	+159
	(+)-Equilenin	14α	+232 E	
11	Methyl 3β-acetoxy-14-iso17-isoetioallocholanate	14β	+91 C	+230
	Methyl 3β-acetoxy-17-iso-etioallocholanate	14α	−139 C	
12	Methyl 14-iso-17-iso-etioallocholanate	14β	+118 C	+220
	Methyl 17-isoetioallocholanate	14α	−102 C	
13	alloPregnane	17β	+52 C	+147
	17-isoalloPregnane	17α	−95 C	

A = acetone, C = chloroform, D = dioxan, E = ethanol.

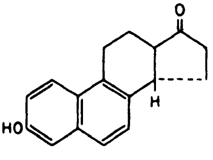
at C_{13} in the presence of an exalting carbonyl group at C_{17} involves a difference $[M^{\beta} - M^{\alpha}]_D$ of +560°; there is good chemical evidence to show that (+)-œstrone and (+)-equilenin correspond in configuration at C_{13}, the angular methyl group being β-orientated. On the provisional assumption that (+)-estrone and (+)-equilenin also correspond in configuration at C_{14} it is possible to write the following set of formulae for (+)-equilenin, and (+)14-isoequilenin, and for (−)-equilenin and (−)14-isoequilenin:



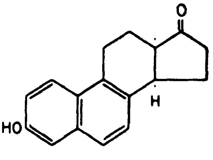
(+)-Equilenin
$[M]_D$ +232°



(+)14-isoEquilenin
+391°



(−)-Equilenin
−232°



(−)14-isoEquilenin
−391°

It will be seen that the molecular rotation difference for inversion at C_{13} is ±620° which is in good agreement with the value $[M^{\beta} - M^{\alpha}]_D$ = +560° given above; for inversion at C_{14}, the molecular rotation difference is ±160°, which, having regard to the adjacent naphthalene ring system is not far from the value $[M^{\beta} - M^{\alpha}]_D$ for C_{14} of +225° (Nos. 11, 12). It may be remarked that no assignment of formulae other than that given above affords agreement.

In principle and subject to the limitations mentioned above (magnitude of $[M^{\beta} - M^{\alpha}]_D$; vicinal action), it should be possible to derive information as to the configuration of any simple nuclear substituent from comparisons of appropriate molecular rotational data. This has been attempted for saturated halides (186), and is particularly useful for hydroxy compounds epimeric at the center bearing the hydroxyl group, owing to the greatly increased spread in the rotations of the derived acetates arising from the increased dissymmetry attending acetylation. Table II shows the molecular rotation differences due to epimeric hydroxyl groups at a single nuclear center.

It will be seen that the differences at C_2 and C_3 are too small to be diagnostic (Nos. 14, 15, 17), but that the differences for acetates (Nos. 16, 18) are more indicative. The considerable differences of opposite sign at C_6 between the *trans*-A/B and *cis*-A/B series (Nos. 21, 22) probably result from vicinal action, and this is to be expected also at C_4 (No. 20). On the other hand there is clearly little vicinal action between C_5 and C_7 with only a single carbon atom interposed (Nos. 23, 26). The effect of nuclear unsaturation is illustrated by one example (No. 24), and the increased dissymmetry resulting from acetylation is

TABLE II

Molecular Rotation Differences Due to Epimeric Hydroxyl Groups at a Single Nuclear Center C_x: x = 2, 3, 4, 6, 7, 11, 12, (15), 16, 17

	Epimerides	OH	$[M]_D$	$[M^\beta - M^\alpha]_D$
14	Cholestan-2-ol	2β	+128 C	−12
	epiCholestan-2-ol	2α	+140 C	
15	Cholestan-3-ol	3β	+93 C	−16
	epiCholestan-3-ol	3α	+109 C	
16	Cholestan-3-ol acetate	3β	+60 C	−69
	epiCholestan-3-ol acetate	3α	+129 C	
17	Coprostan-3-ol	3β	+109 C	−14
	epiCoprostan-3-ol	3α	+123 C	
18	Coprostan-3-ol acetate	3β	+120 C	−86
	epiCoprostan-3-ol acetate	3α	+206 C	
19	3β-Hydroxycholanic acid	3β	+98 E	−22
	Lithocholic acid	3α	+120 E	
20	Cholestan-4-ol, m.p. 189°	4β	+15 C	−97
	epiCholestan-4-ol, m.p. 135°	4α	+112 C	
21	Cholestane-3β:6β-diol	6β	+57 C	−97
	Cholestane-3β:6α-diol	6α	+154 C	
22	3α:6β-Dihydroxycholanic acid	6β	+145 E	+114
	"α"-Hyodeoxycholic acid	6α	+31 E	
23	Cholestane-3β:7β-diol	7β	+214 C	+181
	Cholestane-3β:7α-diol	7α	+33 C	
24	7β-Hydroxycholesterol	7β	+29 C	+380
	7α-Hydroxycholesterol	7α	+351 C	
25	7β-Hydroxycholesteroldiacetate	7β	+252 C	+1099
	7α-Hydroxycholesterol diacetate	7α	−847 C	
26	Ursodeoxycholic acid	7β	+223 E	+180
	Chenodeoxycholic acid	7α	+43 E	
27	3α:11β-Dihydroxycholanic acid	11β	+206 E	+120
	3α:11α-Dihydroxycholanic acid	11α	+86 E	
28	12β-Hydroxycholanic acid	12β	+143 A	−21
	12α-Hydroxycholanic acid	12α	+164 A	
29	12-epiDeoxycholic acid	12β	+151 D	−73
	Deoxycholic acid	12α	+224 E	
30	....	15β	......	......
	....	15α	......	......
31	16-epiEstriol	16β	+254 E	+78
	Estriol	16α	+176 E	
32	Estradiol-3:17β	17β	+220 E	+73
	Estradiol-3:17α	17α	+147 D	
33	Testosterone	17β	+314 E	+108
	epiTestosterone	17α	+206 E	

A = acetone, C = chloroform, D = dioxan, E = ethanol.

again shown (No. 25). Examples of the use of such molecular rotation differences are to be found in numerous papers (see 23, 42, 77, 99, 142, 186, 195, 231).

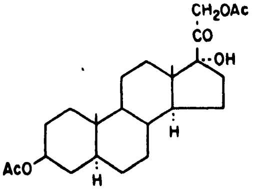
Fieser and Fieser (55) have calculated the molecular rotatory effects of epimeric hydroxyl groups at various centers by comparison of the $[M]_D$ values in Table II with those of a parent deoxy-compound of each pair of epimers. A slightly extended version of their results is given in Table III. A similar table has been given by Barton and Klyne (10).

TABLE III

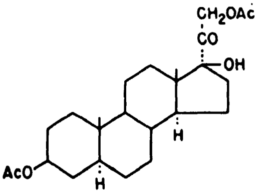
Rotatory Effects of Individual Hydroxyl Groups in Saturated Steroids of the Androstane and 5-Isoandrostane Series

	5α-Series (A/B:*trans*)		5β-Series (A/B:*cis*)	
Position	Δβ−OH	Δα−OH	Δβ−OH	Δα−OH
2	+37	+49	—	—
3	+2	+18	+6	+24
4	−76	+21	—	—
6	−36	+61	+25	−89
7	+121	−60	+103	−77
11			+86	−34
12			+36	+83
15	—	—	—	—
16	+34	−44		
17	−8	−81		

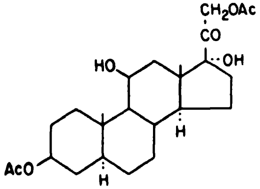
Since vicinal action arising from C_5 does not affect C_7, the effects attributed to hydroxyl groups at C_{11},[12] C_{12},[12] C_{16}, and C_{17} in one series should apply equally to the other.



(XCII) $[M]_D$ +190° (D)



(XCIII) $[M]_D$ −290° (D)



(XCIV) $[M]_D$ +282° (D)

An example of the use of such individual rotatory effects has been given by von Euw and Reichstein (48) in the determination of the configuration at C_{17} of the 3β:11β:17α:21-tetrol diacetate (XCIV). The 17-epimeric 3β:17:21-triol diacetates (XCII), (XCIII) are known, and addition of the molecular rotatory contribution of an 11β-hydroxyl group (+86°, see Table III) to their molecular rotations should give approximately the molecular rotation of (XCIV), determined experimentally as +282°. The values obtained are +276° with (XCII) and −204° with (XCIII); clearly (XCIV) must belong to the 17α-hydroxy series.

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[12] There are some discrepancies between the accepted configurations and the molecular rotation data (64, 232).

Other examples are discussed by Barton and Klyne (10).

d. X-Ray Crystallographic Analysis. The general results of the application of X-ray crystallographic technique to steroids were summarized in 1940 by Bernal *et al.*, (16) and a later review by Dorothy Crowfoot has appeared in Volume II of this publication (39); further work in the field has also been published (136). The detailed analysis of cholesteryl iodide (35) has shown that the iodine atom at C_3 and the side chain at C_{17} are both β-orientated; X-ray examination also indicated that, as is now known on chemical grounds (187), the halogen atoms in cholesteryl chloride and bromide are β-orientated, and that this applies also to 3β-chlorocholestane, formerly known as "α"-cholestanyl chloride.

It has so far not been possible by X-ray examination to verify the configuration of the hydroxyl groups in cholestanol and epicholestanol and in epiandrosterone and androsterone. The evidence is, however, suggestive; the "molecular lengths" (calculated from the slope of the δ-optic axis to the c plane in the crystals) are:

Cholestanol·$2H_2O$	39.5A.	epiCholestanol	34.5A.
epiAndrosterone	12.8A.	Androsterone	11.25A.

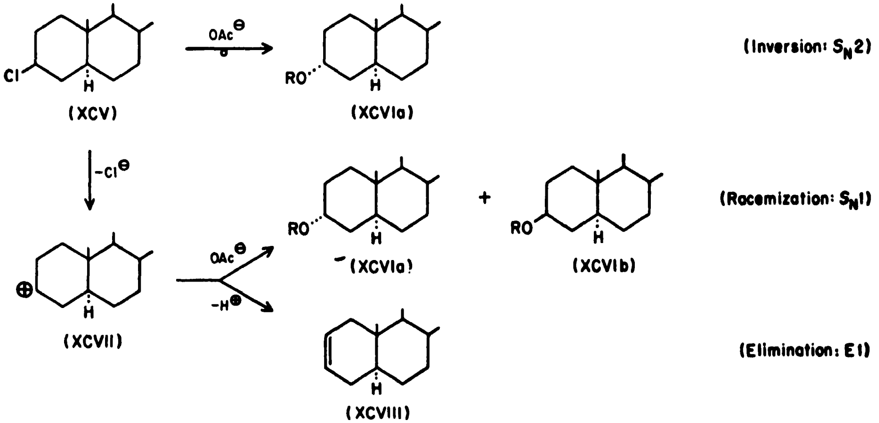
so that, as models suggest, a 3β-hydroxyl group does appear to increase the molecular length (16, 39, 188).

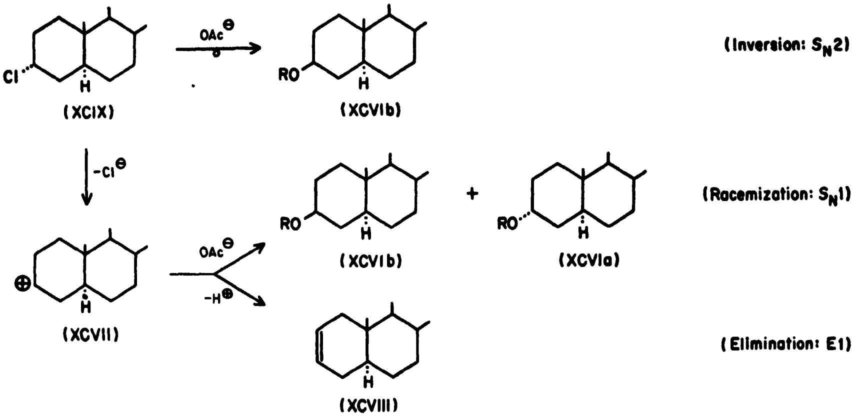
X-ray analysis is facilitated by the presence of an outstandingly heavy atom whose contribution alone largely determines the phases of the X-ray reflections (163); in the absence of such an atom, interpretation of results may be difficult as in the case of cholic acid (66).

e. Examination of Reaction Mechanism. On the basis of the geometrical form and energy of the transition state, it has been concluded that for a bimolecular replacement (S_N2) of one substituent by another at positions 1–4, 6, 7, 11, 12, and probably 15, 16, 17 in saturated derivatives of androstane, allopregnane, allocholane and cholestane, inversion of configuration should occur (186); a similar conclusion may hold for derivatives of 5-isoandrostane, pregnane, cholane, and coprostane and this is under investigation (196). In the presence of suitably placed unsaturated centers or conjugatively unsaturated groups, e.g., in derivatives of androstenes and probably also 5-isoandrostenes, substitution should be accompanied by retention of configuration, the mechanism involved probably being that designated S_N1 (187).

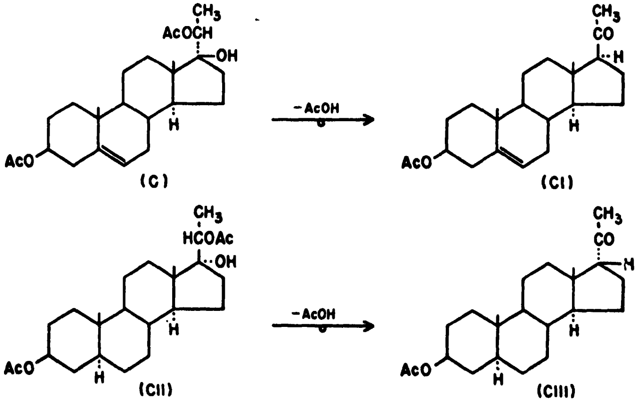
A kinetically controlled examination of a steroid substitution reaction at any of the positions specified above should establish the configuration of the newly introduced substituent if that of the initial material is known. If the reaction is of the first order with respect to each of the two reactants, then the mechanism is bimolecular; but if it is of the first order with respect to only one of the reactants and of zero order with respect to the other, then the mechanism is unimolecular. It should perhaps be pointed out that the total order deduced from the course of the reaction is not necessarily diagnostic of the mechanism. A second order reaction always involves a bimolecular mechanism, but a first order reaction may have either a unimolecular or a bimolecular mechanism. This last possibility arises when one of the reactants is present in so great an excess that there is no significant change in its concentration during the course of the reaction. To distinguish between the two types of substitution, it is best, therefore, to investigate the dependence of the rate on the concentration of each of the two reactants. If, however, it is inconvenient or impossible to find a suitable solvent, less direct methods may be used, as in the work of Winstein and Adams (229). Having found that the reaction of cholesteryl *p*-toluenesulfonate with a large excess of acetic acid is of the first order ($k = 7.9 \times 10^{-3}$ min.$^{-1}$), these authors then showed that the rate constant is 20.0×10^{-3} min.$^{-1}$ in presence of either 0.01 or 0.02 *M* sodium acetate and 24.1×10^{-3} min.$^{-1}$ in the presence of 0.01 *M* lithium perchlorate. Since the acetate ion thus appears to be exerting only a normal salt effect, it was concluded that the reaction is unimolecular. It was thought that in spite of its low concentration the acetate ion would have increased unmistakably the rate of a bimolecular substitution because it is a much more powerful nucleophilic reagent than acetic acid. The positive salt effect also lends support to the conclusion that the mechanism is a unimolecular one, as this type involves a transition state which is much more ionic than the ordinary molecule of the reactant; this conclusion confirms the theoretical prediction previously made (187).

There is a marked lack of examples of kinetically controlled examination of steroid substitution reactions leading to the determination of configuration, probably because of the quantities of material required. This difficulty may be evaded, with but slight decrease in the probable validity of the result, by recourse to a procedure which may be described as a pseudo-kinetic approach. As an example of this, the determination of the configurations of the cholestanyl chlorides (186) may be described. On the basis of extensive kinetic studies it has been shown (38) that in homogeneous substitution of .Hal by .OR in alkyl halides containing besides the halogen, only neutral, saturated groups at the seat of substitution, (*a*) the predominating orientation is inversion of configuration, no matter whether the mechanism is S_N1 or S_N2, (*b*) there is practically complete absence of racemization in mechanism S_N2, whilst extensive racemization is characteristic of mechanism S_N1. The cholestanyl chlorides are typical, if complex, alkyl halides; one of them, m.p. 115° undergoes homogeneous reaction with acetate ions at 180° to afford, after hydrolysis, by chromatographic analysis 3α-hydroxycholestane (XCVIa: R=H), a relatively small quantity of 3β-hydroxycholestane (XCVIb: R=H), and much cholest-2-ene (XCVIII). This chloride is therefore 3β-chlorocholestane (XCV). The 3α-hydroxycholestane arises as the acetate (XCVIa: R=Ac) principally by bimolecular substitution (S_N2) with inversion of configuration. The small quantity of 3β-hydroxycholestane formed as the acetate (XCVIb: R=Ac) results from the operation of the unimolecular mechanism S_N1. Since the tendency of the intermediate cation (XCVII) to become flattened is responsible for racemization, an approximately equivalent quantity of the 3α-epimeride (XCVIa: R=Ac) is derived by this mechanism. Finally the cation (XCVII), instead of combining with an acetate ion, can eject a proton to undergo the elimination reaction E1, giving rise to cholest-2-ene (XCVIII). The epimeric cholestanyl chloride, m.p. 105°, similarly affords the same three products (XCVIa: R=H), (XCVIb: R=H) and (XCVIII), but now the proportion of 3β-hydroxycholestane (XCVIb: R=H) largely exceeds that of the 3α-epimeride (XCVIa: R=H), and this chloride is therefore 3α-chlorocholestane (XCIX). Further examples involving 3-hydrogen sulphates and sulphites of saturated and unsaturated steroids have been described by Lieberman *et al.* (119).

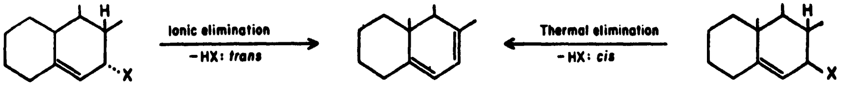




A somewhat different application of reaction mechanism to the determination of configuration is afforded by the Serini reaction (181); here, from consideration of the geometry of the transition state, it was predicted (194) (i) that the 17-isotrioldiacetate (C) would undergo inversion at C_{17} to yield the 17-*n*-ketone (CI), despite the contrary statement of Butenandt *et al.* (30) that the product is the 17-epimeride of (CI), and (ii) that the adreno-cortical substance O diacetate (CII) would furnish the 17-isoketone (CIII) with inversion of configuration. Subsequently, it has been shown that (C) does in fact give (CI) (56) and that (CII) furnishes (CIII) (195).

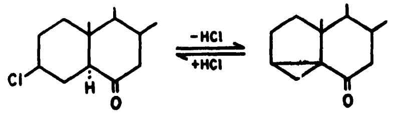


Recently, Barton (9) has suggested that, while *trans*-elimination is characteristic of ionic elimination reactions, preferential *cis*-elimination is the rule in thermal elimination reactions. Application of these considerations can furnish information as to configuration; thus the reactivities of various cholest-7-ene and chol-7-ene derivatives (CIV) (CV) under appropriate conditions indicate configurations at C_7 consistent with those suggested by other evidence, (19, 26, 57, 69, 72, 99, 142, 154, 226, 230, 231). Another example is provided by the extremely ready elimination

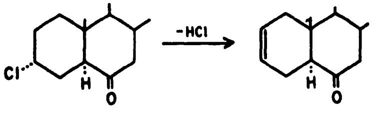


(CIV) (CV)

of hydrogen chloride from the 3β-chloro compound (CVI) to yield *i*-cholestan-6-one (CVII), whereas the 3α-chloro isomeride (CVIII) affords only the unsaturated compound (CIX) with difficulty (42).

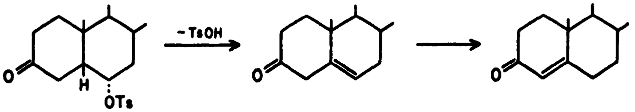


(CVI) (CVII)



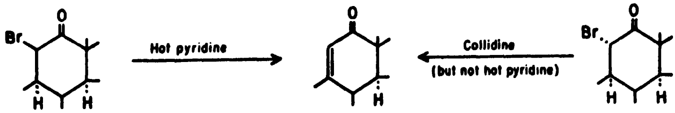
(CVIII) (CIX)

Similarly methyl 3-keto-6α-*p*-tosyloxycholanate (CX) readily undergoes conversion with hot collidine into the rearranged unsaturated ester (CXI) (65). Further examples are furnished by the reactivities to ionic

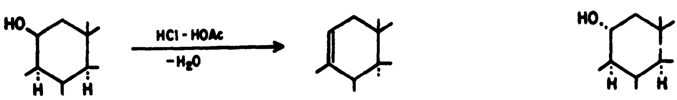


(CX) (CXI)

elimination of epimeric 11-bromoketones (CXII) (CXIII) (62, 180), and of epimeric 11-hydroxy compounds (CXIV) (CXV) (61, 182), the latter being stable to warm dilute mineral acid.

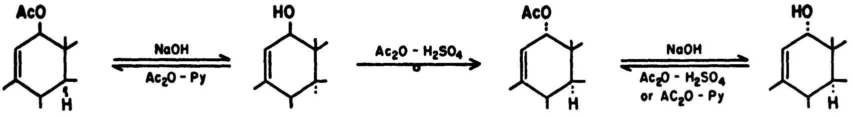


(CXII) (CXIII)



(CXIV) (CXV)

Another application is provided by acid and base catalyzed acetylation. If the acid-catalyzed reaction is a bimolecular substitution and not an esterification, inversion must occur (CXVII → CXVIII); the base-catalyzed reaction would be expected to be an esterification and should proceed with retention of configuration (CXVII → CXVI) (130, 133, 211). It may be noted, however, that with the 12-epimeride (CXIX) of (CXVII) both the acid and base catalyzed reactions are esterifications and proceed with retention of configuration, so that caution is needed in deducing configuration from reaction mechanism unless the latter is known with some certainty. Further, the polar effect of the 9(11)-double bond (which must also lead to flattening of ring C), may here be a factor of which account must be taken.



(CXVI) (CXVII) (CXVIII) (CXIX)

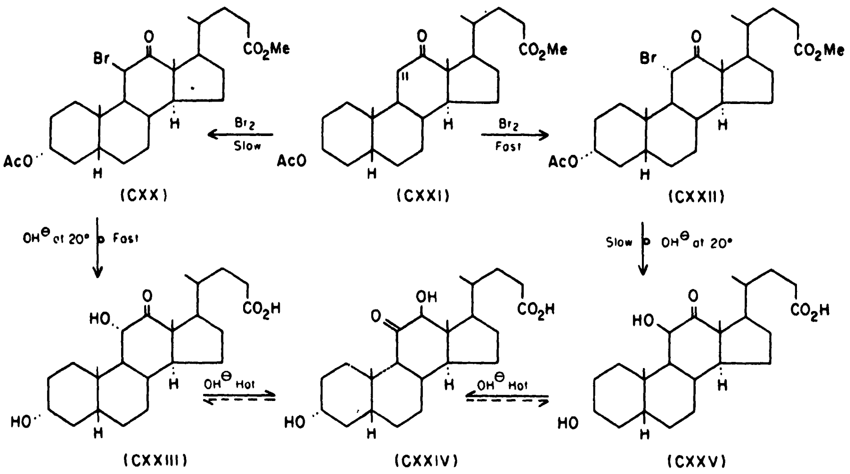
Finally, it may be pointed out that knowledge of the configurations of reactant and reaction product may be used conversely to deduce probable reaction mechanism (193).

f. Examination of Reaction Rates. Polar and steric factors are normally superimposed in bimolecular substitution, but there is no discernible steric effect in unimolecular substitution (44). In steroid bimolecular substitutions S_N2, the proximity of a β-orientated group, as compared with the same α-orientated group, to an angular methyl group or other appropriate molecular feature (e.g., the C_{17}-side chain) should cause a marked difference in reactivity, reflected in reaction velocities. Such rate differences can be used to indicate configuration, but their interpretation requires consideration of the stereochemical character of the reaction involved. Further, when geometrical modification of the form of a ring can occur, rate differences may be dependent on the constellation assumed and unrelated to the conventional configuration normally assigned; an example of this is possibly provided by the hydrolysis rates of the 3-epimeric cholestanyl acetates ($3\beta > 3\alpha$) and coprostanyl acetates ($3\alpha > 3\beta$) (165), whereas for the conventional constellation of ring A in both series, the rates would have been expected to be $3\alpha > 3\beta$ with reference to the effect of the C_{10}-angular methyl group (C_{19}).

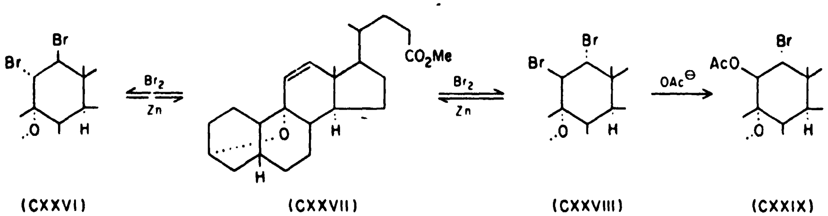
The observation that a 5α-hydroxyl group can be acetylated under forcing conditions (143) is consistent with the configuration assigned; a 5β-hydroxyl group should be strongly hindered by the proximity of C_{19} and possibly of C_4 and C_6.

Comparison of the rates of hydrolysis of the 3β:6α- and 3β:6β-diacetoxycholestanes shows that in the former both acetoxyl groups are split at the same rate, but that in the latter partial hydrolysis to the 6-monoacetate can be achieved, i.e., the reaction rates are 6α > 6β, which is consistent with the proximity of the 6β-hydroxyl group to C_{19} (143). In the case of hyodeoxycholic acid (3α:6α-dihydroxycholanic acid), partial hydrolysis of 3:6-diacetoxy derivatives to 6-monoacetoxy compounds can be achieved with difficulty (65, 78, 125); the epimeric 3α:6β-dihydroxycholanic acid (210) may be capable of hydrolysis as the 3:6-diacetate to the 6-monoacetate, but whether the necessary hindrance by C_{19} can be effective will depend on the constellation assumed by ring B. Similar considerations will clearly apply to 4-hydroxycholestane and 4-hydroxycoprostane derivatives. It may, however, be noted that both 3β:7α- and 3β:7β-dibenzoyloxycholest-5-ene with cold sodium methoxide give 7-monobenzoates, i.e., the reaction rates are 7α = 7β, although the 7β-epimeride should be subject to hindrance by C_{19} (230).

Steric effects are extremely marked at C_{11}; a β-orientated substituent, in contrast to the α-configuration, is greatly hindered by *both* the angular methyl groups at C_{10} and C_{13}. Assignment of configuration at C_{11} on the basis of reactivity can and has been used (160) and has been confirmed subsequently. An 11β-hydroxyl group is either incapable of acetylation or acetylable only with extreme difficulty (202), and 11β-acetoxy compounds (obtained indirectly) are hydrolyzed only under drastic conditions (60, 64), whereas an 11α-hydroxyl group is acetylated and the acetate hydrolyzed with ease i.e., the reactivities are $11\alpha \gg 11\beta$ (62, 63, 64, 232). Similarly the hydrolysis rates of the methyl 3α:11-diacetoxy-12-ketocholanates are 11α > 11β (23). These reactions, though undoubtedly bimolecular in character (S_N2), occur not directly at C_{11} but at the attached oxygen atom and proceed therefore with retention of configuration at C_{11}. By contrast, hydrolysis of the 11-bromides (CXX), (CXXII) with 0.5*N*-potassium hydroxide at 20° takes place at C_{11} itself by mechanism S_N2, proceeds with inversion to give the 11-hydroxy-compounds (CXXIII), (CXXV), and the rates are $11\beta \gg 11\alpha$ (62, 63). Formation of the transition state $OH \cdots C_{11} \cdots Br$ is the rate-determining stage; ease of entry of the attacking hydroxyl ion to the relatively unhindered α-face of C_{11} in (CXX) as compared with the difficulty of access to the strongly hindered β-face of C_{11} in (CXXII) thus determines the rate sequence $11\beta \gg 11\alpha$. Similarly bromination at C_{11} of (CXXI) proceeds slowly to yield little of the 11β-bromide (CXX) but rapidly to give the 11α-bromide (CXXII) as the major product (62, 180). Again, of the two dibromides (CXXVI) (CXXVIII) obtainable from methyl 3α:9α-oxidochol-11-enate (CXXVII) (130) only the



11β:12α-dibromide (CXXVIII) undergoes acetolysis (212). This is surely a bimolecular process (S_N2) and its occurrence is consistent with the foregoing evidence for the rate sequence 11β ≫ 11α; that the replacement affords the 11β-acetoxy compound (CXXIX) is almost certainly due to preservation of configuration at C_{11} by participation of the adjacent bromine atom at C_{12} (cf. 227, 228; cf. also 13). The above rate differences appear to provide good evidence of configuration at C_{11}.[13]



At C_{12}, differential rates of hydrolysis and acetylation of the epimeric 3α:12-dihydroxycholanic acids (108) and methyl 3α:12-diacetoxy-11-ketocholanates (23) indicate the sequence 12β > 12α; the preferential formation of the 12β-hydroxy [Marker-Lawson] acid (CXXIV) (17, 22,

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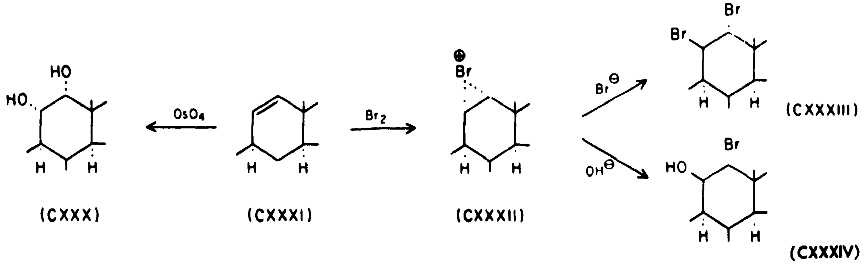
[13] Every known case of reduction of an 11-keto group gives, irrespective of the reducing agent employed, an 11β-hydroxy compound; with lithium aluminium hydride as reducing agent, this is readily understandable since the active entity is the $AlH_4^{\ominus}$ ion which attacks the unhindered α-face of C_{11} in a bimolecular nucleophilic reaction.

64, 124, 232) from either (CXXIII) or (CXXV) (64) also indicates less hindrance at the β-face of C_{12} itself.

Contrariwise, bromination at C_{12} of methyl 3α-acetoxy-11-ketocholanate affords the 12α-bromo compound (131, 212). The addition of bromine or hypobromous acid to methyl 3α-acetoxychol-11-enate (CXXXI) gives a single 11β:12α-dibromide (CXXXIII) (131, cf. 46) or the 11β-hydroxy-12α-bromo derivative (CXXXIV) (137, 155). The established structure (CXXXIV) shows that the first phase of these electrophilic addition reactions is attack at the α-face of C_{12}; since inversion at C_{11} is the consequence, in the second phase of the reactions, of a linear transition state derived from the bromonium ion (CXXXII) or the resonance hybrid to which it contributes (162), the β-orientation of the substituents at C_{11} in (CXXXIII) and (CXXXIV) is inevitable despite steric hindrance. Hydroxylation of (CXXXI) with osmium tetroxide gives 3α:11α:12α-trihydroxycholanic acid (CXXX) (232).

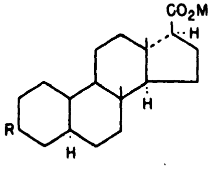


(CXXX) (CXXXI) (CXXXII) (CXXXIII) (CXXXIV)

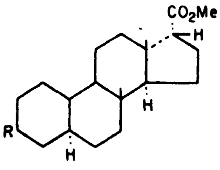
These reactions appear to indicate less hindrance at the α-face of C_{12} than at the β-face, but with some inconsistencies; these contradictory appearances are possibly related to the fixed chair constellation of ring C in saturated *trans*-C/D compounds, which is modified by flattening on introduction of unsaturation at $C_{9(11)}$ and especially at $C_{11(12)}$. It has been suggested that the observed hindrance of 12α-hydroxyl or acetoxyl groups is due to the C_{17}-side chain rather than to the C_{18} angular methyl group, and it has been observed that the rates of hydrolysis of 12α-acetates increase as the side chain is shortened (108). It must be concluded that differential reaction rates do not form a reliable guide to configuration at C_{12}.

At C_{16}, relative rates of alkaline hydrolysis (S_N2) of epimeric acetoxy compounds were used to deduce configuration (77); the inequality found, 16α > 16β, was ascribed to the influence of the β-orientated side chain at C_{17}.

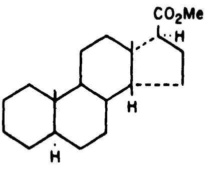
At C_{17}, differential rates of alkaline hydrolysis (S_N2) of epimeric acetoxy-compounds (testosterone and dihydrotestosterone acetates) were used to assign configuration (165); on the supposition that only a 17β-acetoxyl could be hindered by C_{18}, the less rapidly hydrolyzed epimers were assigned the 17β-configuration. Optical rotatory evidence suggests however that this assignment must be reversed (68); also there is a large body of evidence which indicates that in 17α-hydroxy-20-keto steroids [natural 17*n*-series] the hydroxyl group cannot be acetylated although the β-orientated 20-carbonyl group is not specially hindered, whereas in 17β-hydroxy-20-keto steroids [17-iso series] the hydroxyl group is capable of acetylation but that the α-orientated 20-carbonyl group is strongly hindered (49), i.e., the reactivity sequence is 17β > 17α. It appears that in compounds with *trans*-C/D ring union, the steric effect of the C_{12}-methylene group on a 17α-hydroxyl group is more important than is that of the angular methyl group (C_{18}) on a 17β-hydroxyl group. These considerations, applying as they do to reactions at an oxygen atom attached to C_{17}, will also hold for reactions at a carbon atom one place removed from C_{17}, i.e., C_{20}; thus, as suggested by examination of molecular models, the 17β-carboxylic ester (CXXXV) and the 17α-carboxylic ester (CXXXVI) are hydrolyzed with potassium hydroxide (mechanism S_N2) under the same conditions to the extent of 48% and 18% (CXXXV, CXXXVI: R=H), (171) and of 52% and 26.4% (CXXXV, CXXXVI: R=OAc), respectively (49). Clearly, the different stereochemical pattern present in *cis*-C/D ring compounds will lead to different mutual effects at C_{17} and C_{12}; molecular models suggest that the unknown 14-iso-17β-carboxylic ester (CXXXVII) and the 14-iso-17α-carboxylic ester (CXXXVIII) should both be readily hydrolyzed (171). Steric hindrance effects at C_{17} and at an adjacent atom (oxygen or C_{20}) have been surveyed by Fieser and Fieser (54, 55); they conclude that it seems to be an empirical fact that whereas the rear side of the steroid nucleus offers better opportunity for attack at C_{17}, the front side has more space available for accommodation of a substituent, and subsequent work tends to confirm this conclusion (175).



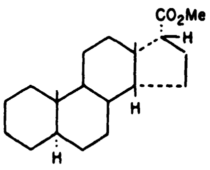
(CXXXV)



(CXXXVI)



(CXXXVII)



(CXXXVIII)

In the D-homoandrostane series, initial rates of hydrolysis of epimeric 17a-acetates and ease of acetylation of the epimeric 17a-hydroxy compounds have been employed to assign provisional configurations at C_{17a} (184).

V. CONFIGURATION IN THE SIDE CHAIN

1. *Orientation of the Side Chain*

The complete X-ray analysis of cholesteryl iodide by Carlisle and Crowfoot (35) clearly indicated that the 17-*normal* side chain is β-orientated. Investigation of the capacity for 21 → 12-lactone formation of the various bisnordeoxycholic acids enabled Sorkin and Reichstein (197) (see p. 270) to show that the spatial relationship between the 12-hydroxyl group and the side chain must be 12α:17β or 12β:17α but did not permit them to distinguish between these possibilities. Finally, Sorkin and Reichstein (199) (see p. 269) by examination of the capacity for 17 → 12-lactone formation of all the four possible etiodeoxycholic acids epimeric-at C_{12} and C_{17} proved conclusively that the side chain possesses the β-configuration in etiodeoxycholic acid and in the natural steroids.

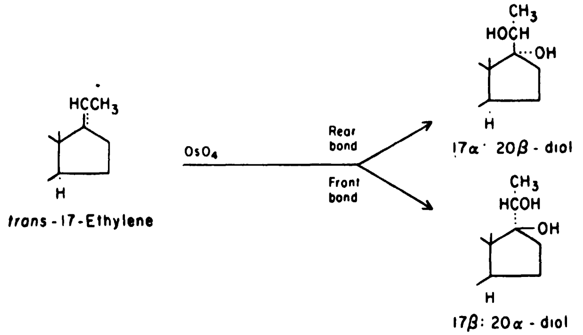
The 17-iso configuration of the side chain present in the cardiac "alloglycosides" and "alloaglycones" (200), properly termed 17-isoglycosides and 17-isoaglycones, appears to result by the action of enzymes on the 17-*n*-glycosides. Numerous compounds with the unnatural 17-iso side chain have been obtained synthetically.

2. *Configuration at C_{20} Relative to C_{17}*

Owing to "free" rotation of the C_{17}—C_{20}-bond, stereoisomerism in the side chain is of the classical tartaric acid type. A definite orientation cannot be assigned, although favored constellations, probably of the staggered type, must exist for given substituents. Discussion here will be confined to substituent hydroxyl groups, although pairs of stereoisomerides involving other groups are known (25, 166, 197).

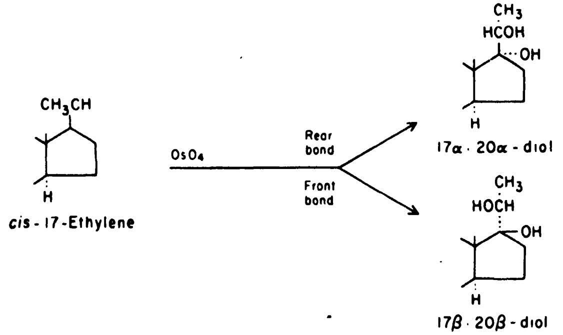
Reichstein and Prins (152, cf. 47) originally used an arbitrary nomenclature to designate C_{20}-isomerides, but pointed out that their use of the symbols 20α, 20β implied no agreement with the equally arbitrary system previously adopted by Marker *et al.* (122).

Fieser and Fieser (54, 55) have discussed the relationships between the hitherto arbitrarily designated configurations at C_{20} and the established configurations at C_{17}. Their convention, illustrated by the chart, depends on the *cis*-addition of osmium tetroxide to 17-ethylenes to give, *via* cyclic osmic esters, 17:20-diols (CXXXIX, CXL, CXLI, CXLII), and involves the rotation, in a molecular model, of C_{20} so that C_{21} is at the *back* of the molecule in the "reading position":



(CXXXIX)

(CXL)



(CXLI)

(CXLII)

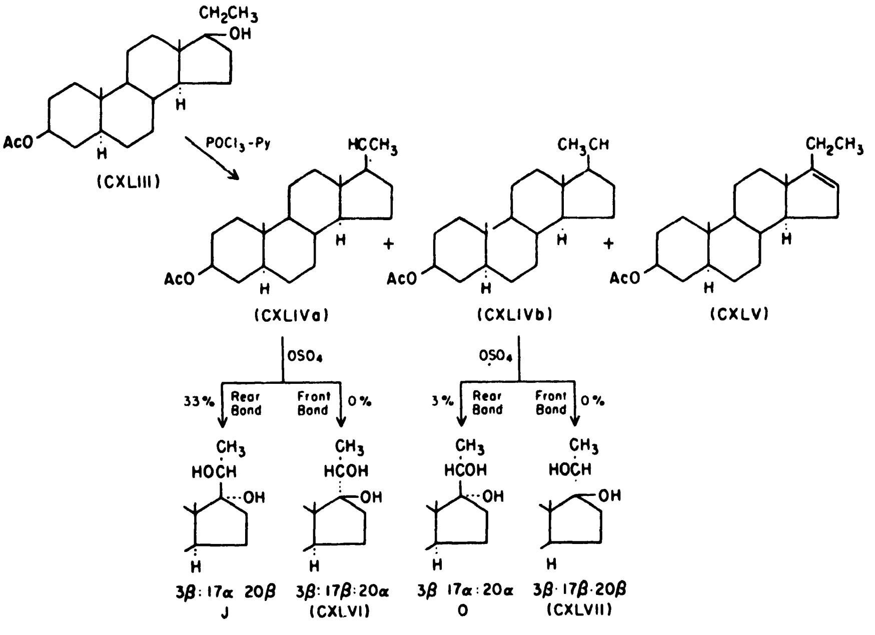
In respect of C_{20}, this convention agrees with that of E. Fischer for the sugars; C_{17} does not need to be considered in this connection, because configuration at C_{17} is defined in another way and because the Fischer convention is employed only for unbranched carbon chains.

Application of the convention of Fieser and Fieser to the assignment of configuration at C_{20} depends on knowledge (*a*) of the orientation of the side-chain in the resulting diol, (*b*) of the geometrical character [*trans-* or *cis:* C_{18}-Me/C_{20}-Me] of the 17-ethylene; (*a*) can be determined experimentally, and for (*b*) since direct evidence is not yet available, the reasonable assumption is made that dehydration of a suitable carbinol proceeds more readily to give a *trans*-17-ethylene than a *cis*-17-ethylene, so that a *trans*-olefin should predominate in the reaction mixture (28, 29, 109, 177).[14]

[14] Sarett (176, 177, 178) has shown that hydroxylation of a crude mixture of 17- and 20-unsaturated 3α-acetoxy-11-ketopregnenes affords not only 17α:20β- but also 17α:20α-diols, which indicates the presence of relatively much *cis*-17-ethylene.

It may also be noted that hydroxylation of 21-hydroxypregn-17-enes invariably leads to only one of the 17α-hydroxy-compounds out of the four theoretically possible glycerol-derivatives. Only single stereochemically pure 21-acyloxypregn-17-enes appear to arise by anionotropy of 17-iso-17β-acyloxypregn-20-enes; since the inter-

The convention may be exemplified by reference to the partial synthesis of the adrenocortical substances J and O (158). Dehydration of 17-isoallopregnane-3β:17β-diol 3-monoacetate (CXLIII) affords a mixture of *trans*- (CXLIVa) and *cis*-pregn-17-enes (CXLIVb), (probably containing also the pregn-16-ene (CXLV) (175)), which, by hydroxylation with osmium tetroxide, yields two of the four possible triols; these are substances J and O, which are known to be 20-epimerides of the 17-*n*-series (73). The proportions of the *trans*- and *cis*-ethylenes in the mixture used thus appear to be about 10:1. One of the missing isomerides, 17-isoallopregnane-3β:17β:20α-triol (CXLVI), has been prepared in another way (152). The other missing isomeride, 17-isoallopregnane-3β:17β:20β-triol (CXLVII), was originally described as the diacetate, m.p. 135°, $[\alpha]_D$ − 18° (158), which has been shown to be an equimolecular compound of substance J diacetate and another diacetate (probably that of allopregnane-3β:16α:17α-triol derived from (CXLV)) (175). The true 3β:17β:20β-triol (CXLVII) has recently been described by Salamon and Reichstein (175). Optical rotatory evidence supports the configurations assigned in the 17*n*-series (11, 55) since it is found generally that a 20β-acetoxysteroid is more dextrorotatory than the 20α-epimeride;



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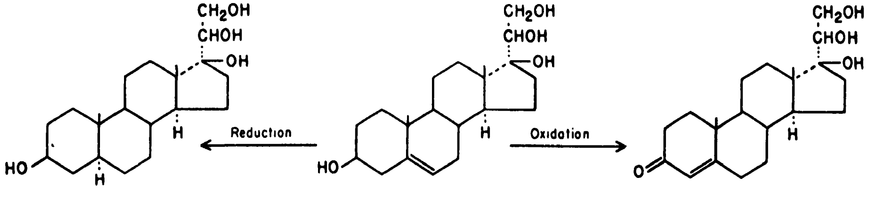
mediate mesomeric cations should in principle be able to give rise to both *cis*- and *trans*-17-ethylenes, the products are probably the *trans*-isomerides.

this relationship does not seem to hold in the 17-iso-series [see p. 298] possibly because "free rotation" of the C_{17}—C_{20}-bond is more strongly hindered.

The convention of Fieser and Fieser leads to configurations at C_{20} for substances J and O in agreement with the arbitrary indices of Prins and Reichstein (152), who originally called J a 3β:17β:20β-triol, later altered following recognition of the β-orientation of the side-chain to 3β:17α:20β, and designated O as a 3β:17β:20α-triol, later amended to 3β:17α:20α. Although J and O are the reference compounds adopted by Reichstein for configuration at C_{20}, because his assignments of configuration at C_{20} have in general been arbitrary except where two or more compounds have been shown to belong to the same stereochemical series, the nomenclature of Fieser and Fieser will not necessarily correspond with that of Reichstein (cf. 11). Thus (CXLVI) was originally called a 3β:17α:20β-triol by Prins and Reichstein (152), a name revised by Salamon and Reichstein (174) to 3β:17β:20β, and finally amended by Salamon and Reichstein (175) to 3β:17β:20α to correspond with the Fieser convention. Similarly, the true triol (CXLVII) recently prepared by Salamon and Reichstein (175) is now designated as 3β:17β:20β.

Salamon and Reichstein (174, 175) have been able to correlate the 20-epimeric 17-iso triols (CXLVI), (CXLVII) with the three pairs of 20-epimeric 17-iso tetrols represented by (CXLVIIIa and b), (XLIXa and b), and (CLa and b), which have themselves been correlated by oxidation and reduction.



(CXLVIII a and b) (CXLIX a and b) (CL a and b)

17-Isoallopregn-20-ene-3β:17β-diol 3-monoacetate (CLI) with perbenzoic acid affords the epimeric oxides (CLIIa and b); these are reduced to the triols (CXLVI) and (CXLVII) respectively with lithium aluminium hydride, and hydrolysed to the tetrols (CXLVIIIa) and (CXLVIIIb) respectively with potassium hydroxide. The configuration at C_{20} of the tetrol (CXLVIIIb) is further secured by its re-conversion *via* the 3-benzoate-21-tosylate to the oxide (CLIIa). The following table, reproduced with slight modification and with the kind permission of Professor and Mrs. Fieser, sets out the molecular rotations of various 20-epimers

TABLE IV

Molecular Rotations of 20-Epimers

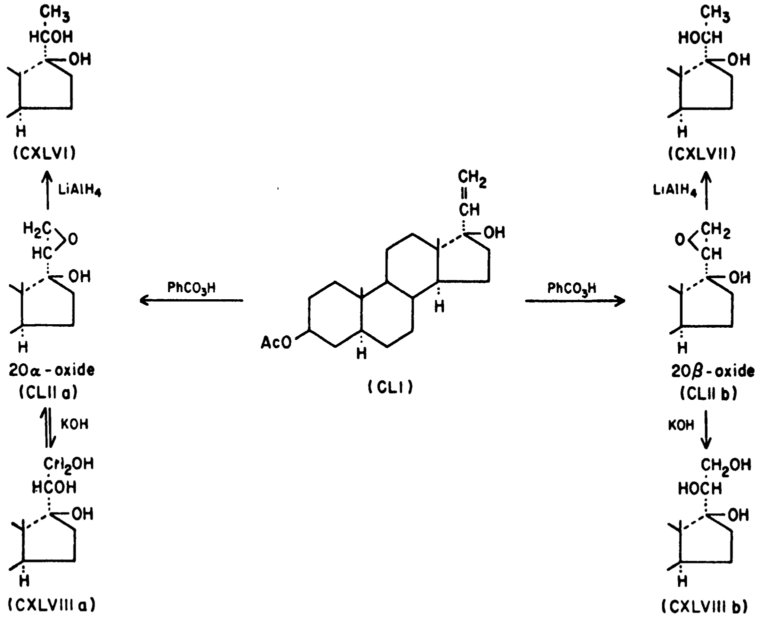
Type	Compound	[M$_D$]		Δ^{Ac}
		Alcohol	Acetate	
20β-17-*normal*-Series				
17α:20β:21-(OH)$_3$	Substance K	−4 E	+258 A	(+262)
	Substance A	+87 A	+326 A	+239
	Substance E	+317 E	+730 A	(+413)
	Substance U	+508 A	+800 A	+292
	Pregn-4-enetriol-3-one	+220 D	+541 D	+321
20β:21-(OH)$_2$	Substance T	+610 A	+732 A	+122
	Pregnanediol 3:11-dione	+214 A	+320 A	+106
17α:20β-(OH)$_2$	Substance J	−27 E	+103 A	(+130)
	5-Dehydro-J	−251 E	−140 E	+111
20α-17-*normal*-Series				
17α:20α:21-(OH)$_3$	Epi-K		−5 A	
20α:21-(OH)$_2$	Epi-T	+612 A	+594 A	−18
	Pregnanediol-3:11-dione	+239 A	+195 A	−44
17α:20α-(OH)$_2$	Substance O	−43 E	−132 A	(−89)
20β-17-*iso*-Series				
17β:20β:21-(OH)$_3$	(CXLVIIIb)	0 E	0 A	(0)
	(CXLIXb)		−210 A	
	(CLb)	+229 D	+189 D	−40
17β:20β-(OH)$_2$	(CXLVII)	−17 E	−67 A	(−50)
	5-Dehydro-deriv. of (CXLVII)*	−341 E	−310 E	+31
20α-17-*iso*-Series				
17β:20α:21-(OH)$_3$	(CXLVIIIa)	0 E	−153 A	(−153)
	(CXLIXa)	−256 D	−422 D	−166
	(CLa)		+96 A	
17β:20α-(OH)$_2$	(CXLVI)	−30 E	−46 C	(−16)

A = acetone, C = chloroform, D = dioxan, E = ethanol.

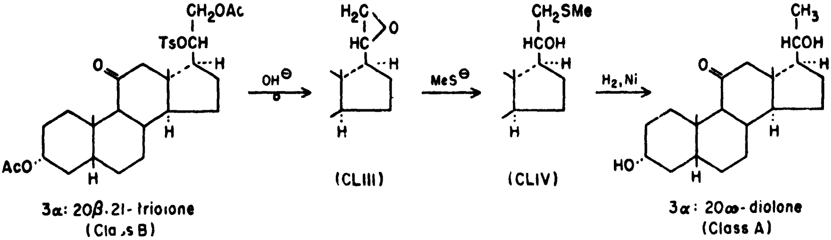
Figures representing comparisons in different solvents and involving uncertainties estimated at ± 40 units are enclosed in brackets.

*** The diacetate of this reputed substance (30,152) is probably a molecular compound of 5-dehydro-J diacetate and pregn-5-ene-3β:16α:17α-triol 3:16-diacetate (175), in which case Δ^{Ac} is here meaningless.**

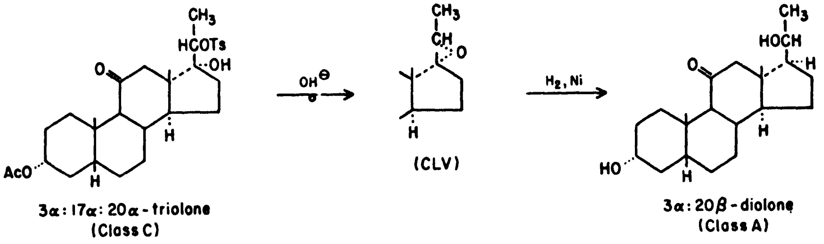
and the molecular rotation differences (Δ^{Ac}) between the free triols and tetrols and their di- and triacetates. In the natural series, acetylation of 20β-triols and 20β-diols leads to positive increments Δ^{Ac} of 200–400 and 100 units respectively; in each instance the increment Δ^{Ac} for the 20α-epimeride is of opposite sign and is 150–200 units more negative. From a survey of the chemical and rotatory evidence, Fieser and Fieser (55) were able to deduce that of Reichstein's seven adrenocortical substances asymmetric at C_{20}, six substances [A, E, J, K, T, and U] belong to the 20β-hydroxy-series; the seventh substance [O] is the 20α-epimeride of substance J. In the unnatural series, the increment Δ^{Ac} appears to be negative for both the 20β- and 20α-hydroxy-series; there is a lack of consistency between configuration assigned on a chemical basis and optical rotatory evidence (11, 55, 159, 175, 178); nevertheless the "Rule of Shift" (59) may find application here, though possibly in some manner not necessarily related to that in the 17-*n*-series. Despite apparent optical anomalies it seems probable that the interpretation given by Fieser and Fieser (55) is correct.



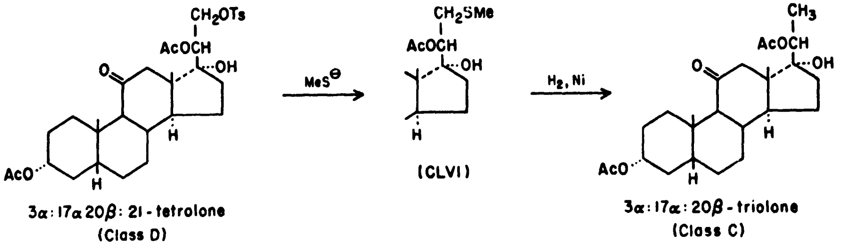
In the 17-*normal*-11-ketopregnane series, Sarett (176, 177, 178) has been able to correlate 20-epimeric 3α:20-diols (Class A), 3α:20:21-triols (Class B), 3α:17α:20-triols (Class C), and 3α:17α:20:21-tetrols (Class D); within each class the 3α:20α- and 3α:20β-compounds were linked with the corresponding 3-keto derivatives. The method used involves elimination of a 20-*p*-tosyl group by treatment with alkali; this is known (139, 213) to occur with inversion of configuration. The resulting 20:21-oxide (CLIII) is split with sodium methyl mercaptide to furnish the thioether (CLIV), also obtained directly from the original 20β-tosyl-21-acetate, which is reduced with Raney nickel:



Alternatively, the 17.20-oxide (CLV) can be reduced directly when only a single isomeride is formed:



Finally, a 21-*p*-tosyl group can be eliminated by treatment with sodium methyl mercaptide and reduction of the resulting thioether (CLVI):

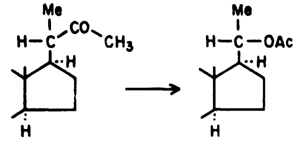


The assignments of configuration so made are confirmed by use of molecular rotation differences between the 20-epimerides and their acetates. It appears that these differences are not appreciably affected by 17α-hydroxyl or a 21-hydroxyl group.

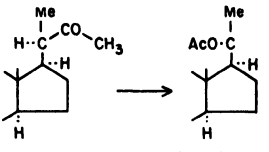
On the assumption that such molecular rotation differences are qualitatively independent of the presence of an 11-keto group or the configuration of the 3-hydroxyl group, it can be shown that substances J and O fall respectively into Sarett's 20β- and 20α-series. Thus all assignments of configuration made by Sarett (178) correspond with the convention of Fieser and Fieser (55).

3. Configuration at C_{20} Nonrelative to C_{17}

The convention of Fieser and Fieser, discussed in the previous section, relates configuration at C_{20} to that at C_{17}, primarily in 17:20-diols; as pointed out, this has been extended by Sarett (178) to embrace certain 20-hydroxy-compounds of the 17-*n*-series bearing an α-orientated hydrogen atom at C_{17}. In principle, it should ultimately be possible to describe all 20-hydroxy-steroids in terms of the Fieser and Fieser convention, using the indices 20α and 20β. Some progress can indeed be made; thus the combined results of Butenandt and Schmidt-Thomé (31) and of Prins and Reichstein (152) relate substance J (allopregnane-3β:17α:20β-triol) to the diol described by Marker *et al.* (122) as allopregnane-3β:20β-diol.[15] In Marker's arbitrary system, 20-hydroxysteroids occurring as urinary metabolites are designated 20α [e.g., "pregnanediol" of human pregnancy urine ≡ pregnane-3α:20α-diol], and their epimers, produced predominately but generally not exclusively by catalytic reduction of 20-ketosteroids, are named 20β. Thus it appears that Marker's system is fortuitously in agreement with the convention of Fieser and Fieser, and optical rotatory evidence in support of this conclusion has been adduced (11, 178).



20-*n*-(A) 3β:20α-diol (C)



20-iso (B) 3β:20β-diol (D)

Some few pairs of 20-epimerides are known; their configuration at C_{20} cannot yet be related to that at C_{17},[16] but can be correlated between individuals. Such compounds may be distinguished by the arbitrary designations 20a, 20b [Fieser and Fieser (55)] or 20-*n*, 20-*iso* [Reichstein (197)]. Each of four 20-*n*-derivatives of 3β-hydroxynorcholanic acid (140), and three of four 20-*n*-derivatives of the 3α:12-dihydroxybisnorcholanic acids (197, 198) are more dextrorotatory than the corresponding 20-iso compounds; they thus fall into two series and it seems possible that the acidic grouping of the 20-*n*-series, and hence the terminal parts of the sterol and bile acid side chain are β-orientated (55).[17] Cholesterol, ergosterol, stigmasterol, and the bile acids correspond in configuration at C_{20} and are provisionally to be described as 20b-compounds; stellastanol appears to belong to the 20a-series (14).

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[15] This relationship is confirmed by conversion of substance J diacetate into Marker's allopregnane-3β:20β-diol diacetate (private communication from Dr. D. A. Prins, Ciba Inc., Summit, N. J.).

[16] It has recently been shown that the 20-*n*- and 20-iso-3β-acetoxy-22-ketonorallocholanes (A), (B) by oxidation with peracetic or perbenzoic acid afford the diacetates of allopregnane-3β:20α-diol (C) and allopregnane-3β:20β-diol (D) respectively (private communication from Dr. E. W. Meyer, The Glidden Co., Chicago 39, Ill.).

4. Nonrelative Configuration at C_{24}

Stereoisomerism at C_{24} is encountered among the higher sterols and the derived stanols. The assignment of individuals to the 24a or 24b series[18] has been achieved by chemical correlation of individuals and by application of molecular rotation differences, (a) to various stanol derivatives, and (b) to the determination of the configurations of the aldehydes and ketones obtained from the side chain by oxidation.

Table V illustrates method (a) and is reproduced by kind permission of Professor and Mrs. Fieser.

TABLE V

Molecular Rotation Differences for Higher Stanols and Derivatives

Sterol	Side chain subst.	$[M_D]$ ($CHCl_3$)				$[M_D]$ (Deriv)-$[M_D]$ (Sterol)		
		Sterol	Acetate	Benzoate	Ketone	Δ^{Ac}	Δ^{Bz}	Δ^{Ket}
Cholestanol......	None	+93	+60	+98	+158	−33	+5	+65
Ergostanol.......	24b-CH_3	+64	+27		+140	−37		+76
Campestanol.....	24a-CH_3	+125	+80			−45		
Stellastanol......	24a-CH_3	+88	+62			−26		
Stigmastanol.....	24b-C_2H_5	+100	+69	+104	+170	−31	+4	+70
Poriferastanol....	24a-C_2H_5	+90	+70		+171	−20		+81
"γ"-Sitostanol...	24a-C_2H_5	+83	+46		+157	−37		+74

Ergostanol is thus 24b-methylcholestanol, and campestanol is 24a-methylcholestanol (52), whilst stigmastanol [≡ "β"-sitostanol] is 24b-ethylcholestanol and poriferastanol [≡ ? "γ"-sitostanol] is 24aethylcholestanol (15, 41). Evaluation of the rotatory contributions of 24-alkyl groups confirms the serial assignments made (55).

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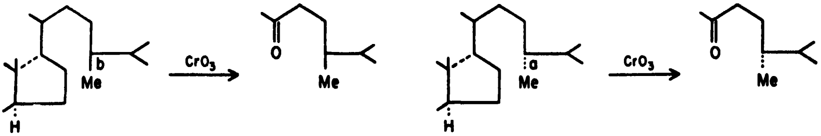
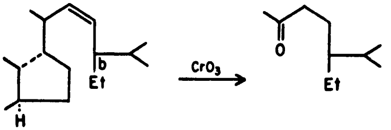
[17] Recent results obtained by P. Wieland and K. Miescher (*Helv. Chim. Acta*, 1949, **32**, 1922) however suggest the opposite conclusion.

[18] These indices reverse those proposed by Bergmann and Low (15).



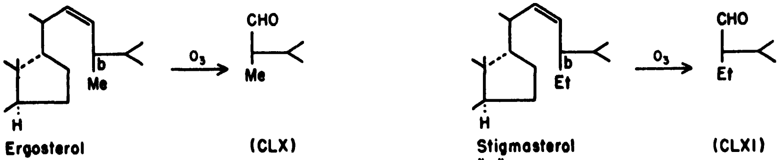
Ergostanol (CLVII) Campesterol (CLVIII)

Method (b) may be illustrated by the following examples. Oxidation with chromium trioxide of ergostanol (71) and campesterol (52) gives (−)-5:6-dimethylheptan-2-one (CLVII) and (+)-5:6-dimethylheptan-2-one (CLVIII) respectively; these compounds are therefore 24-stereoisomerides. "β"-Sitosterol affords (+)-5-ethyl-6-methylheptan-2-one (CLIX) (41):



"β"-Sitosterol (CLIX)

The opposite signs of the molecular rotations of the homologous ketones (CLVII), (CLIX) and their semicarbazones do not indicate opposite configuration at C_{24}. This sign difference is predicted by application (55) of Marker's empirical rule (121), and Bergmann and Low (15) have shown that the differences in the molecular rotations of the semicarbazones and 2:4-dinitrophenylhydrazones of the homologous aldehydes (CLX), (CLXI) obtained from ergosterol (71, 161) and brassicasterol (51), and stigmasterol (70) and "α"-spinasterol (57) respectively by ozonolysis are of the same sign and of the same order. Ergosterol,



Ergosterol
Brassicasterol (CLX) Stigmasterol
"α"-Spinasterol (CLXI)

brassicasterol, "β"-sitosterol [≡ 22:23-dihydrostigmasterol], stigmasterol, and "α"-spinasterol thus all belong to the 24b-series, whilst campesterol belongs to the 24a-series.

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